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(54) Title: AN ENZYME CAPABLE OF DEGRADING CELLULOSE OR HEMICELLULOSE

(a)

1 Xaa Xaa Gln Cys Gly Gly Xaa Xaa Xaa Xaa Gly Xaa Xaa Xaa Cys Xaa
 10
 Xaa Xaa Xaa Xaa Cys Xaa Xaa Xaa Asn Xaa Xaa Tyr Xaa Gln Cys Xaa
 20
 Xaa Xaa -
 30
 Xaa

(57) Abstract

A cellulose- or hemicellulose-degrading enzyme which is derivable from a fungus other than *Trichoderma* or *Phanerochaete*, and which comprises a carbohydrate binding domain homologous to a terminal A region of *Trichoderma reesei* cellulases, which carbohydrate binding domain comprises amino acid sequence (α) or a subsequence thereof capable of effecting binding of the enzyme to an insoluble cellulosic or hemicellulosic substrate.

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AN ENZYME CAPABLE OF DEGRADING CELLULOSE OR HEMICELLULOSE

FIELD OF INVENTION

5 The present invention relates to a cellulose- or hemicellulose-degrading enzyme, a DNA construct coding for the enzyme, a method of producing the enzyme, and an agent for degrading cellulose or hemicellulose comprising the enzyme.

10 BACKGROUND OF THE INVENTION

Enzymes which are able to degrade cellulose have previously been suggested for the conversion of biomass into liquid fuel, gas and feed protein. However, the production of fermentable 15 sugars from biomass by means of cellulolytic enzymes is not yet able to compete economically with, for instance, the production of glucose from starch by means of α -amylase due to the inefficiency of the currently used cellulolytic enzymes. Cellulolytic enzymes may furthermore be used in the brewing 20 industry for the degradation of β -glucans, in the baking industry for improving the properties of flour, in paper pulp processing for removing the non-crystalline parts of cellulose, thus increasing the proportion of crystalline cellulose in the pulp, and in animal feed for improving the digestibility of 25 glucans. A further important use of cellulolytic enzymes is for textile treatment, e.g. for reducing the harshness of cotton-containing fabrics (cf., for instance, GB 1 368 599 or US 4,435,307), for soil removal and colour clarification of fabrics (cf., for instance, EP 220 016) or for providing a 30 localized variation in colour to give the fabrics a "stone-washed" appearance (cf., for instance, EP 307 564).

The practical exploitation of cellulolytic enzymes has, to some extent, been set back by the nature of the known cellulase 35 preparations which are often complex mixtures of a variety of single cellulase components, and which may have a rather low specific activity. It is difficult to optimise the production

of single components in multiple enzyme systems and thus to implement industrial cost-effective production of cellulolytic enzymes, and their actual use has been hampered by difficulties arising from the need to employ rather large quantities of the 5 enzymes to achieve the desired effect.

The drawbacks of previously suggested cellulolytic enzymes may be remedied by using single-component enzymes selected for a high specific activity.

10

Single-component cellulolytic enzymes have been isolated from, e.g. Trichoderma reesei (cf. Teeri et al., Gene 51, 1987, pp. 43-52; P.M. Abuja, Biochem. Biophys. Res. Comm. 156, 1988, pp. 180-185; and P.J. Kraulis, Biochemistry 28, 1989, pp. 7241-15 7257). The T. reesei cellulases have been found to be composed of a terminal A region responsible for binding to cellulose, a B region linking the A region to the core of the enzyme, and a core containing the catalytically active domain. The A region of different T. reesei cellulases has been found to be highly 20 conserved, and a strong homology has also been observed with a cellulase produced by Phanerochaete chrysosporium (Sims et al., Gene 74, 1988, pp. 411-422).

SUMMARY OF THE INVENTION

25

It has surprisingly been found that other fungi, which are not closely related to either Trichoderma reesei or Phanerochaete chrysosporium, are capable of producing enzymes which contain a region which is homologous to the A region of T. reesei 30 cellulases.

Accordingly, the present invention relates to a cellulose- or hemicellulose-degrading enzyme which is derivable from a fungus other than Trichoderma or Phanerochaete, and which comprises a 35 carbohydrate binding domain homologous to a terminal A region of Trichoderma reesei cellulases, which carbohydrate binding domain comprises the following amino acid sequence

1 Xaa Xaa Gln Cys Gly Gly Xaa Xaa Xaa Xaa Gly Xaa Xaa Xaa Cys Xaa
20
5 Xaa Xaa Xaa Xaa Cys Xaa Xaa Xaa Asn Xaa Xaa Tyr Xaa Gln Cys Xaa
Xaa

10 or a subsequence thereof capable of effecting binding of the enzyme to an insoluble cellulosic or hemicellulosic substrate. "Xaa" is intended to indicate variations in the amino acid sequence of the carbohydrate binding domain of different enzymes. A hyphen is intended to indicate a "gap" in the amino 15 acid sequence (compared to other, similar enzymes).

In the present context, the term "cellulose" is intended to include soluble and insoluble, amorphous and crystalline forms of cellulose. The term "hemicellulose" is intended to include 20 glucans (apart from starch), mannans, xylans, arabinans or polyglucuronic or polygalacturonic acid. The term "carbohydrate binding domain" ("CBD") is intended to indicate an amino acid sequence capable of effecting binding of the enzyme to a carbohydrate substrate, in particular cellulose or 25 hemicellulose as defined above. The term "homologous" is intended to indicate a high degree of identity in the sequence of amino acids constituting the carbohydrate binding domain of the present enzyme and the amino acids constituting the A region found in T. reesei cellulases ("A region" is the term 30 used to denote the cellulose (i.e. carbohydrate) binding domain of T. reesei cellulases).

It is currently believed that cellulose- or hemicellulose-degrading enzymes which contain a sequence of amino acids which 35 is identifiable as a carbohydrate binding domain (or "A region" based on its homology to the A region of T. reesei cellulases possess certain desirable characteristics as a result of the function of the carbohydrate binding domain in the enzyme molecule which is to mediate binding to solid substrates 40 (including cellulose) and consequently to enhance the activity

of such enzymes towards such substrates. The identification and preparation of carbohydrate binding domain-containing enzymes from a variety of microorganisms is therefore of considerable interest.

5

Cellulose- or hemicellulose-degrading enzymes of the invention may conveniently be identified by screening genomic or cDNA libraries of different fungi with a probe comprising at least part of the DNA encoding the A region of T. reesei cellulases.

10 Due to the intraspecies (i.e. different T. reesei cellulases) and interspecies homology observed for the carbohydrate binding domains of different cellulose- or hemicellulose-degrading enzymes, there is reason to believe that this screening method constitutes a convenient way of isolating enzymes of current
15 interest.

DETAILED DISCLOSURE OF THE INVENTION

Carbohydrate binding domain (CBD) containing enzymes of the
20 invention may, in particular, be derivable from strains of Humicola, e.g. Humicola insolens, Fusarium, e.g. Fusarium oxysporum, or Myceliophthora, e.g. Myceliophthora thermophile.

Some of the variations in the amino acid sequence shown above
25 appear to be "conservative", i.e. certain amino acids are preferred in these positions among the various CBD-containing enzymes of the invention. Thus, in position 1 of the sequence shown above, the amino acid is preferentially Trp or Tyr. In position 2, the amino acid is preferentially Gly or Ala. In
30 position 7, the amino acid is preferentially Gln, Ile or Asn. In position 8, the amino acid is preferentially Gly or Asn. In position 9, the amino acid is preferentially Trp, Phe or Tyr. In position 10, the amino acid is preferentially Ser, Asn, Thr or Gln. In position 12, the amino acid is preferentially Pro,
35 Ala or Cys. In position 13, the amino acid is preferentially Thr, Arg or Lys. In position 14, the amino acid is preferentially Thr, Cys or Asn. In position 18, the amino acid

is preferentially Gly or Pro. In position 19, the amino acid (if present) is preferentially Ser, Thr, Phe, Leu or Ala. In position 20, the amino acid is preferentially Thr or Lys. In position 24, the amino acid is preferentially Gln or Ile. In 5 position 26, the amino acid is preferentially Gln, Asp or Ala. In position 27, the amino acid is preferentially Trp, Phe or Tyr. In position 29, the amino acid is preferentially Ser, His or Tyr. In position 32, the amino acid is preferentially Leu, Ile, Gln, Val or Thr.

10

Examples of specific CBD-containing enzymes of the invention are those which comprise one of the following amino acid sequences

15 Trp Gly Gln Cys Gly Gly Gln Gly Trp Asn Gly Pro Thr Cys Cys Glu
Ala Gly Thr Thr Cys Arg Gln Gln Asn Gln Trp Tyr Ser Gln Cys
Leu;

Trp Gly Gln Cys Gly Gly Ile Gly Trp Asn Gly Pro Thr Thr Cys Val
20 Ser Gly Ala Thr Cys Thr Lys Ile Asn Asp Trp Tyr His Gln Cys
Leu;

Trp Gly Gln Cys Gly Gly Ile Gly Phe Asn Gly Pro Thr Cys Cys Gln
Ser Gly Ser Thr Cys Val Lys Gln Asn Asp Trp Tyr Ser Gln Cys
25 Leu;

Trp Gly Gln Cys Gly Gly Asn Gly Tyr Ser Gly Pro Thr Thr Cys Ala
Glu Gly - Thr Cys Lys Lys Gln Asn Asp Trp Tyr Ser Gln Cys Thr
Pro;

30

Trp Gly Gln Cys Gly Gly Gln Gly Trp Gln Gly Pro Thr Cys Cys Ser
Gln Gly - Thr Cys Arg Ala Gln Asn Gln Trp Tyr Ser Gln Cys Leu
Asn;

35 Trp Gly Gln Cys Gly Gly Gln Gly Tyr Ser Gly Cys Thr Asn Cys Glu
Ala Gly Ser Thr Cys Arg Gln Gln Asn Ala Tyr Tyr Ser Gln Cys
Ile;

Trp Gly Gln Cys Gly Gly Gln Gly Tyr Ser Gly Cys Arg Asn Cys Glu Ser Gly Ser Thr Cys Arg Ala Gln Asn Asp Trp Tyr Ser Gln Cys Leu;

Trp Ala Gln Cys Gly Gly Asn Gly Trp Ser Gly Cys Thr Thr Cys Val 5 Ala Gly Ser Thr Cys Thr Lys Ile Asn Asp Trp Tyr His Gln Cys Leu;

Trp Gly Gln Cys Gly Gly Gln Asn Tyr Ser Gly Pro Thr Thr Cys Lys Ser Pro Phe Thr Cys Lys Lys Ile Asn Asp Phe Tyr Ser Gln Cys 10 Gln; or

Trp Gly Gln Cys Gly Gly Asn Gly Trp Thr Gly Ala Thr Thr Cys Ala Ser Gly Leu Lys Cys Glu Lys Ile Asn Asp Trp Tyr Tyr Gln Cys Val

15 The cellulose- or hemicellulose-degrading enzyme of the invention may further comprise an amino acid sequence which defines a linking B region (to use the nomenclature established for T. reesei cellulases) adjoining the carbohydrate binding domain and connecting it to the catalytically active domain of 20 the enzyme. The B region sequences established so far for enzymes of the invention indicate that such sequences are characterized by being predominantly hydrophilic and uncharged, and by being enriched in certain amino acids, in particular glycine and/or asparagine and/or proline and/or serine and/or 25 threonine and/or glutamine. This characteristic structure of the B region imparts flexibility to the sequence, in particular in sequences containing short, repetitive units of primarily glycine and asparagine. Such repeats are not found in the B region sequences of T. reesei or P. chrysosporium which contain 30 B regions of the serine/threonine type. The flexible structure is believed to facilitate the action of the catalytically active domain of the enzyme bound by the A region to the insoluble substrate, and therefore imparts advantageous properties to the enzyme of the invention.

35

Specific examples of B regions contained in enzymes of the invention have the following amino acid sequences

Ala Arg Thr Asn Val Gly Gly Ser Thr Gly Gly Gly Asn Asn Gly
Gly Gly Asn Asn Gly Gly Asn Pro Gly Gly Asn Pro Gly Gly Asn Pro
Gly Gly Asn Pro Gly Gly Asn Pro Gly Gly Asn Pro Gly Gly Asn Cys
Ser Pro Leu;

5

Pro Gly Gly Asn Asn Asn Pro Pro Pro Ala Thr Thr Ser Gln Trp
Thr Pro Pro Pro Ala Gln Thr Ser Ser Asn Pro Pro Pro Thr Gly Gly
Gly Gly Asn Thr Leu His Glu Lys;

10

Gly Gly Ser Asn Asn Gly Gly Asn Asn Asn Gly Gly Asn Asn
Asn Gly Gly Gly Asn Asn Asn Gly Gly Asn Asn Asn Gly Gly
Gly Asn Thr Gly Gly Ser Ala Pro Leu;

15 Val Phe Thr Cys Ser Gly Asn Ser Gly Gly Ser Asn Pro Ser Asn
Pro Asn Pro Pro Thr Pro Thr Phe Ile Thr Gln Val Pro Asn Pro
Thr Pro Val Ser Pro Pro Thr Cys Thr Val Ala Lys;

20 Pro Ala Leu Trp Pro Asn Asn Asn Pro Gln Gln Gly Asn Pro Asn Gln
Gly Gly Asn Asn Gly Gly Asn Gln Gly Gly Asn Gly Gly Cys
Thr Val Pro Lys;

25 Pro Gly Ser Gln Val Thr Thr Ser Thr Ser Ser Ser Thr Thr
Ser Arg Ala Thr Ser Thr Ser Ala Gly Gly Val Thr Ser Ile Thr
Thr Ser Pro Thr Arg Thr Val Thr Ile Pro Gly Gly Ala Ser Thr Thr
Ala Ser Tyr Asn;

30 Glu Ser Gly Gly Asn Thr Asn Pro Thr Asn Pro Thr Asn Pro Thr
Asn Pro Thr Asn Pro Thr Asn Pro Trp Asn Pro Gly Asn Pro Thr Asn
Pro Gly Asn Pro Gly Gly Asn Gly Gly Asn Gly Asn Cys Ser
Pro Leu; or

35 Pro Ala Val Gln Ile Pro Ser Ser Ser Thr Ser Ser Pro Val Asn Gln
Pro Thr Ser Thr Ser Thr Ser Thr Ser Thr Ser Ser Pro Pro
Val Gln Pro Thr Thr Pro Ser Gly Cys Thr Ala Glu Arg

In another aspect, the present invention relates to a carbohydrate binding domain homologous to a terminal A region of *Trichoderma reesei* cellulases, which carbohydrate binding domain comprises the following amino acid sequence

5

1 Xaa Xaa Gln Cys Gly Gly Xaa Xaa Xaa Xaa Gly Xaa Xaa Xaa Cys Xaa
 10
 20 Xaa Xaa Xaa Xaa Cys Xaa Xaa Xaa Asn Xaa Xaa Tyr Xaa Gln Cys Xaa
 30
 -
 Xaa

15 or a subsequence thereof capable of effecting binding of a protein to an insoluble cellulosic or hemicellulosic substrate.

Examples of specific carbohydrate binding domains are those with the amino acid sequence indicated above.

20

In a further aspect, the present invention relates to a linking B region derived from a cellulose- or hemicellulose-degrading enzyme, said region comprising an amino acid sequence enriched in the amino acids glycine and/or asparagine and/or proline 25 and/or serine and/or threonine and/or glutamine. As indicated above, these amino acids may often occur in short, repetitive units. Examples of specific B region sequences are those shown above.

30 The present invention provides a unique opportunity to "shuffle" the various regions of different cellulose- or hemicellulose-degrading enzymes, thereby creating novel combinations of the CBD, B region and catalytically active domain resulting in novel activity profiles of this type of
35 enzymes. Thus, the enzyme of the invention may be one which comprises an amino acid sequence defining a CBD, which amino acid sequence is derived from one naturally occurring cellulose- or hemicellulose-degrading enzyme, an amino acid sequence defining a linking B region, which amino acid sequence
40 is derived from another naturally occurring cellulose- or

hemicellulose-degrading enzyme, as well as a catalytically active domain derived from the enzyme supplying either the CBD or the B region or from a third enzyme. In a particular embodiment, the catalytically active domain is derived from an 5 enzyme which does not, in nature, comprise any CBD or B region. In this way, it is possible to construct enzymes with improved binding properties from enzymes which lack the CBD and B regions.

10 The enzyme of the invention is preferably a cellulase such as an endoglucanase (capable of hydrolysing amorphous regions of low crystallinity in cellulose fibres), a cellobiohydrolase (also known as an exoglucanase, capable of initiating degradation of cellulose from the non-reducing chain ends by 15 removing cellobiose units) or a β -glucosidase.

In a still further aspect, the present invention relates to a DNA construct which comprises a DNA sequence encoding a cellulose- or hemicellulose-degrading enzyme as described 20 above.

A DNA sequence encoding the present enzyme may, for instance, be isolated by establishing a cDNA or genomic library of a microorganism known to produce cellulose- or hemicellulose- 25 degrading enzymes, such as a strain of Humicola, Fusarium or Mycelopthora, and screening for positive clones by conventional procedures such as by hybridization to oligonucleotide probes synthesized on the basis of the full or partial amino acid sequence of the enzyme or probes based on the partial or full 30 DNA sequence of the A region from T. reesei cellulases, as indicated above, or by selecting for clones expressing the appropriate enzyme activity, or by selecting for clones producing a protein which is reactive with an antibody raised against a native cellulose- or hemicellulose-degrading enzyme.

35

Alternatively, the DNA sequence encoding the enzyme may be prepared synthetically by established standard methods, e.g.

the phosphoamidite method described by S.L. Beaucage and M.H. Caruthers, Tetrahedron Letters 22, 1981, pp. 1859-1869, or the method described by Matthes et al., The EMBO J. 3, 1984, pp. 801-805. According to the phosphoamidite method, 5 oligonucleotides are synthesized, e.g. in an automatic DNA synthesizer, purified, annealed, ligated and cloned in appropriate vectors.

Finally, the DNA sequence may be of mixed genomic and 10 synthetic, mixed synthetic and cDNA or mixed genomic and cDNA origin prepared by ligating fragments of synthetic, genomic or cDNA origin (as appropriate), the fragments corresponding to various parts of the entire DNA construct, in accordance with standard techniques. Thus, it may be envisaged that a DNA 15 sequence encoding the CBD of the enzyme may be of genomic origin, while the DNA sequence encoding the B region of the enzyme may be of synthetic origin, or vice versa; the DNA sequence encoding the catalytically active domain of the enzyme may conveniently be of genomic or cDNA origin. The DNA 20 construct may also be prepared by polymerase chain reaction using specific primers, for instance as described in US 4,683,202 or R.K. Saiki et al., Science 239, 1988, pp. 487-491.

The present invention also relates to an expression vector 25 which carries an inserted DNA construct as described above. The expression vector may suitably comprise appropriate promotor, operator and terminator sequences permitting the enzyme to be expressed in a particular host organism, as well as an origin of replication enabling the vector to replicate in the host 30 organism in question.

The resulting expression vector may then be transformed into a suitable host cell, such as a fungal cell, a preferred example of which is a species of Aspergillus, most preferably 35 Aspergillus oryzae or Aspergillus niger. Fungal cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of

the cell wall in a manner known per se. The use of Aspergillus as a host microorganism is described in EP 238,023 (of Novo Industri A/S), the contents of which are hereby incorporated by reference.

5

Alternatively, the host organisms may be a bacterium, in particular strains of Streptomyces and Bacillus, and E. coli. The transformation of bacterial cells may be performed according to conventional methods, e.g. as described in 10 Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, 1989.

The screening of appropriate DNA sequences and construction of vectors may also be carried out by standard procedures, cf. 15 Sambrook et al., op. cit.

The invention further relates to a method of producing a cellulose- or hemicellulose-degrading enzyme as described above, wherein a cell transformed with the expression vector of 20 the invention is cultured under conditions conducive to the production of the enzyme, and the enzyme is subsequently recovered from the culture. The medium used to culture the transformed host cells may be any conventional medium suitable for growing the host cells in question. The expressed enzyme 25 may conveniently be secreted into the culture medium and may be recovered therefrom by well-known procedures including separating the cells from the medium by centrifugation or filtration, precipitating proteinaceous components of the medium by means of a salt such as ammonium sulphate, followed 30 by chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

By employing recombinant DNA techniques as indicated above, techniques of fermentation and mutation or other techniques 35 which are well known in the art, it is possible to provide cellulose- or hemicellulose-degrading enzymes of a high purity and in a high yield.

The present invention further relates to an agent for degrading cellulose or hemicellulose, the agent comprising a cellulose- or hemicellulose-degrading enzyme as described above. It is contemplated that, dependent on the specificity of the enzyme, 5 it may be employed for one (or possibly more) of the applications mentioned above. In a particular embodiment, the agent may comprise a combination of two or more enzymes of the invention or a combination of one or more enzymes of the invention with one or more other enzymes with cellulose- or 10 hemicellulose-degrading activity.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the construction of plasmid p SX224;
Fig. 2 shows the construction of plasmid pHW485;
15 Fig. 3 shows the construction of plasmid pHW697 and pHW704;
Fig. 4 shows the construction of plasmid pHW768;
Fig. 5 is a restriction map of plasmid pSX320;
Fig. 6 shows the construction of plasmid pSX777
Fig. 7 shows the construction of plasmid pCaHj170;
20 Fig. 8 shows the construction of plasmid IM4;
Fig. 9 shows the SOE fusion of the ~43kD endoglucanase signal peptide and the N-terminal of Endo1;
Fig. 10 shows the construction of plasmid pCaHj180;
Fig. 11 shows the DNA sequence and derived amino acid sequence
25 of F.oxysporum C-family cellobiohydrolase;
Fig. 12 shows the DNA sequence and derived amino acid sequence of F.oxysporum F-family cellulase;
Fig. 13 shows the DNA sequence and derived amino acid sequence of F.oxysporum C-family endoglucanase;
30 Fig. 14.A-E shows the DNA sequence and derived amino acid sequence of H.insolens endoglucanase 1(EG1); and
Fig. 15A-D shows the DNA sequence and derived amino acid sequence of a fusion of the B.lautus (NCIMB 40250) Endo 1 catalytic domain and the CBD and B region of H.insolens ~43kD 35 endoglucanase.

The invention is further illustrated in the following examples which are not in any way intended to limit the scope of the invention as claimed.

5 Example 1

Isolation of A region-containing clones from *H. insolens*

From *H. insolens* strain DSM 1800 (described in, e.g. WO
10 89/09259) grown on cellulose, mRNA was prepared according to
the method described by Koplan et al., Biochem. J. 183 (1979)
181-184. A cDNA library containing 20,000 clones was obtained
substantially by the method of Okayama and Berg, Methods in
Enzymology 154, 1987, pp. 3-28.

15

The cDNA library was screened as described by Gergen et al.,
Nucl. Acids Res. 7(8), 1979, pp. 2115-2136, with
oligonucleotide probes in the antisense configuration, designed
according to the published sequences of the N-terminal part of
20 the A-region of the four *T. reesei* cellulase genes (Penttilä et
al., Gene 45 (1986), 253-63; Saloheimo et al., Gene 63, (1988),
11-21; Shoemaker et al., Biotechnology, October 1983, 691-696;
Teeri et al., Gene 51 (1987) 43-52. The probe sequences were as
follows:

25

NOR-804	5'-CTT GCA CCC GCT GTA CCC AAT GCC ACC GCA CTG CCC
(~ EG 1)	CCA-3'
NOR-805	5'-CGT GGG GCC GCT GTA GCC AAT ACC GCC GCA CTG GCC
(~CBH 1)	GTA-3'
30 NOR-807	5'-AGT CGG ACC CGA CCA ATT CTG GCC ACC ACA TTG GCC
(~CBH 2)	CCA-3'
NOR-808	5'-CGT AGG TCC GCT CCA ACC AAT ACC TCC ACA CTG GCC
(~EG 3)	CCA-3'

35 Screening yielded a large number of candidates hybridising well
to the A-region probes. Restriction mapping reduced the number
of interesting clones to 17, of which 8 have so far been

sequenced (as described by Haltiner et al., Nucl. Acids Res. 13, 1985, pp. 1015-1025) sufficiently to confirm the presence of a terminal CBD as well as a B-region.

5 The deduced amino acid sequences obtained for the CBDs were as follows

A-1: Trp Gly Gln Cys Gly Gly Gln Gly Trp Asn Gly Pro Thr Cys
Cys Glu Ala Gly Thr Thr Cys Arg Gln Gln Asn Gln Trp Tyr Ser Gln
10 Cys Leu;

A-5: Trp Gly Gln Cys Gly Gly Ile Gly Trp Asn Gly Pro Thr Thr
Cys Val Ser Gly Ala Thr Cys Thr Lys Ile Asn Asp Trp Tyr His Gln
Cys Leu;

15

CBH-2: Trp Gly Gln Cys Gly Gly Ile Gly Phe Asn Gly Pro Thr Cys
Cys Gln Ser Gly Ser Thr Cys Val Lys Gln Asn Asp Trp Tyr Ser Gln
Cys Leu;

20

A-8: Trp Gly Gln Cys Gly Gly Asn Gly Tyr Ser Gly Pro Thr Thr
Cys Ala Glu Gly - Thr Cys Lys Lys Gln Asn Asp Trp Tyr Ser Gln
Cys Thr Pro;

A-9: Trp Gly Gln Cys Gly Gly Gln Gly Trp Gln Gly Pro Thr Cys
25 Cys Ser Gln Gly - Thr Cys Arg Ala Gln Asn Gln Trp Tyr Ser Gln
Cys Leu Asn;

A-11: Trp Gly Gln Cys Gly Gly Gln Gly Tyr Ser Gly Cys Thr Asn
Cys Glu Ala Gly Ser Thr Cys Arg Gln Gln Asn Ala Tyr Tyr Ser Gln
30 Cys Ile;

A-19: Trp Gly Gln Cys Gly Gly Gln Gly Tyr Ser Gly Cys Arg Asn
Cys Glu Ser Gly Ser Thr Cys Arg Ala Gln Asn Asp Trp Tyr Ser Gln
35 Cys Leu; and

-43 kd: Trp Ala Gln Cys Gly Gly Asn Gly Trp Ser Gly Cys Thr Thr Cys Val Ala Gly Ser Thr Cys Thr Lys Ile Asn Asp Trp Tyr His Gln Cys Leu

5 The deduced amino acid sequences obtained for the B region were as follows

A1: Ala Arg Thr Asn Val Gly Gly Ser Thr Gly Gly Asn Asn Gly Gly Asn Asn Gly Gly Asn Pro Gly Gly Asn Cys Ser Pro Leu;

A5: Pro Gly Gly Asn Asn Asn Asn Pro Pro Pro Ala Thr Thr Ser Gln Trp Thr Pro Pro Pro Ala Gln Thr Ser Ser Asn Pro Pro Pro Thr Gly Gly Gly Asn Thr Leu His Glu Lys;

A8: Gly Gly Ser Asn Asn Gly Gly Asn Asn Asn Gly Gly Asn Asn Asn Gly Gly Gly Asn Asn Asn Gly Gly Asn Asn Asn Gly Gly Asn Thr Gly Gly Ser Ala Pro Leu;

20 A11: Val Phe Thr Cys Ser Gly Asn Ser Gly Gly Ser Asn Pro Ser Asn Pro Asn Pro Pro Thr Pro Thr Phe Ile Thr Gln Val Pro Asn Pro Thr Pro Val Ser Pro Pro Thr Cys Thr Val Ala Lys;

25 A19: Pro Ala Leu Trp Pro Asn Asn Asn Pro Gln Gln Gly Asn Pro Asn Gln Gly Gly Asn Asn Gly Gly Asn Gln Gly Gly Asn Gly Gly Cys Thr Val Pro Lys;

CBH2: Pro Gly Ser Gln Val Thr Thr Ser Thr Thr Ser Ser Ser 30 Thr Thr Ser Arg Ala Thr Ser Thr Ser Ala Gly Gly Val Thr Ser Ile Thr Thr Ser Pro Thr Arg Thr Val Thr Ile Pro Gly Gly Ala Ser Thr Thr Ala Ser Tyr Asn;

A9: Glu Ser Gly Gly Asn Thr Asn Pro Thr Asn Pro Thr Asn 35 Pro Thr Asn Pro Thr Asn Pro Thr Asn Pro Trp Asn Pro Gly Asn Pro Thr Asn Pro Gly Asn Pro Gly Gly Asn Gly Gly Asn Cys Ser Pro Leu; or

Pro Ala Val Gln Ile Pro Ser Ser Ser Thr Ser Ser Pro Val Asn Gln
Pro Thr Ser Thr Ser Thr Ser Thr Ser Thr Ser Pro Pro
Val Gln Pro Thr Thr Pro Ser Gly Cys Thr Ala Glu Arg

5

Example 2

Expression in *A. oryzae* of a CBH 2-type cellulase from *H. insolens*

10

The complete sequence of one of the CBD clones shows a striking similarity to a cellobiohydrolase (CBH 2) from *T. reesei*.

The construction of the expression vector pSX224 carrying the 15 *H. insolens* CBH 2 gene for expression in and secretion from *A. oryzae* is outlined in Fig. 1. The vector p777 containing the pUC 19 replicon and the regulatory regions of the TAKA amylase promoter from *A. oryzae* and glucoamylase terminator from *A. niger* is described in EP 238 023. pSX 217 is composed of the 20 cloning vector pcDV1-pL1 (cf. Okayama and Berg, op. cit.) carrying the *H. insolens* CBH 2 gene on a 1.8 kb fragment. The CBH 2 gene contains three restriction sites used in the construction: A BalI site at the initiating methionine codon in the signal sequence, a BstBI site 620 bp downstream from the 25 BalI site and an AvaII site 860 bp downstream from the BstBI site. The AvaII site is located in the non-translated C-terminal part of the gene upstream of the poly A region, which is not wanted in the final construction. Nor is the poly G region upstream of the gene in the cloning vector. This region 30 is excised and replaced by an oligonucleotide linker which places the translational start codon close to the BamHI site at the end of the TAKA promoter.

The expression vector pSX 224 was transformed into *A. oryzae* 35 IFO 4177 using the amdS gene from *A. nidulans* as the selective marker as described in EP 238 023. Transformants were grown in YPD medium (Sherman et al., Methods in Yeast Genetics, Cold

Spring Harbor Laboratory, 1981) for 3-4 days and analysed for new protein species in the supernatant by sodium dodecyl sulphate polyacrylamide gel electrophoresis. The CBH 2 from *H. insolens* formed a band with an apparent Mw of 65 kD indicating 5 a substantial glycosylation of the protein chain, which is calculated to have a Mw of 51 kD on the basis of the amino acid composition. The intact enzyme binds well to cellulose, while enzymatic degradation products of 55 kD and 40 kD do not bind, indicating removal of the A-region and possibly the B-region.

10 The enzyme has some activity towards filter paper, giving rise to release of glucose. As expected, it has very limited endoglucanase activity as measured on soluble cellulose in the form of carboxy methyl cellulose.

15 Example 3

Isolation of *Fusarium oxysporum* genomic DNA

A freeze-dried culture of *Fusarium oxysporum* was reconstituted 20 with phosphate buffer, spotted 5 times on each of 5 FOX medium plates (6% yeast extract, 1.5% K₂HPO₄, 0.75% MgSO₄ 7H₂O, 22.5% glucose, 1.5% agar, pH 5.6) and incubated at 37°C. After 6 days of incubation the colonies were scraped from the plates into 15 ml of 0.001% Tween-80 which resulted in a thick and cloudy 25 suspension.

Four 1-liter flasks, each containing 300 ml of liquid FOX medium, were inoculated with 2 ml of the spore suspension and were incubated at 30°C and 240 rpm. On the 4th day of 30 incubation, the cultures were filtered through 4 layers of sterile gauze and washed with sterile water. The mycelia were dried on Whatman filter paper, frozen in liquid nitrogen, ground into a fine powder in a cold mortar and added to 75 ml of fresh lysis buffer (10 mM Tris-Cl 7.4, 1% SDS, 50 mM EDTA, 35 100 µl DEPC). The thoroughly mixed suspension was incubated in a 65°C waterbath for 1 hour and then spun for 10 minutes at 4000 rpm and 5°C in a bench-top centrifuge. The supernatant

was decanted and EtOH precipitated. After 1 hour on ice the solution was spun at 19,000 rpm for 20 minutes. The supernatant was decanted and isopropanol precipitated. Following centrifugation at 10,000 rpm for 10 minutes, the supernatant 5 was decanted and the pellets allowed to dry.

One milliliter of TER solution (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 100 µg RNase A) was added to each tube, and the tubes were stored at 4°C for two days. The tubes were pooled and 10 placed in a 65°C waterbath for 30 minutes to suspend non-dissolved DNA. The solution was extracted twice with phenol/CHCl₃/isoamyl alcohol, twice with CHCl₃/isoamyl alcohol and then ethanol precipitated. The pellet was allowed to settle and the EtOH was removed. 70% EtOH was added and the DNA stored 15 overnight at -20°C. After decanting and drying, 1 ml of TER was added and the DNA was dissolved by incubating the tubes at 65°C for 1 hour. The preparation yielded 1.5 mg of genomic DNA.

**Amplification, cloning and sequencing of DNA amplified with
20 degenerate primers**

To amplify DNA from C-family (according to the nomenclature of Henrissat et al. *Gene* 81 (1), 1989, pp. 83-96) cellulases using PCR (cf. US 4,683,195 and US 4,683,202) each "sense" 25 oligonucleotide was used in combination with each "antisense" oligonucleotide. Thus, the following primer pair was used:

Primer 1

ZC3220

Primer 2

ZC3221

30

ZC3220: GCC AAC TAC GGT ACC GG(A/C/G/T) TA(C/T) TG(C/T)
GA(C/T) (A/G/T) (C/G) (A/G/C/T) CA(G/A) TG

ZC3221: GCG TTG GCC TCT AGA AT(G/A) TCC AT(C/T) TC(A/G/C/T)
35 (C/G/T) (A/T) (G/A) CA(G/A) CA

In the PCR reaction, 1 µg of Fusarium oxysporum genomic DNA was used as the template. Ten times PCR buffer is 100mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM MgCl₂, 0.1% gelatin (Perkin-Elmer Cetus). The reactions contained the following ingredients:

5

	dH ₂ O	35.75 µl
	10X PCR buffer	5 µl
	template DNA	5 µl
	primer 1	2 µl (40pmol)
10	primer 2	2 µl (40 pmol)
	<u>Tag polymerase</u>	0.25 µl (1.25 U)
	total	50 µl

The PCR reactions were performed for 40 cycles under the
15 following conditions:

94 °C	1.5 min
45°	2.0 min
72°	2.0 min

20 Five microliters of each reaction was analyzed by agarose gel electrophoresis. The sizes of the DNA fragments were estimated from DNA molecular weight markers. The reacton primed with ZC3220 and ZC3221, produced two DNA fragments of appropriate size to be candidates for fragments of C-family cellulases. The
25 agarose sections containing these two fragments were excised, and the DNA was electroeluted and digested with the restriction enzymes KpnI and Zbal. The fragments were ligated into the vector pUC18 which had been cut with the same two restriction enzymes. The ligation were transformed into E. coli and mini-
30 prep DNA was prepared from the resulting colonies. The DNA sequences of these inserts were determined and revealed that two new C-family cellulases had been identified, one a new cellobiohydrolase and the other a new endoglucanase.

The PCR cloning strategy described above for the C-family
35 cellulases was applied using other primers which encoded conserved cellulase sequences within the known F-family

cellulases (cf. Henrissat et al., op. cit.) The following primer pair was used for amplification of Fusarium genomic DNA.

	<u>Primer 1</u>	<u>Primer 2</u>
5	ZC3226	ZC3227
	<u>ZC3226:</u> TCC TGA CGC CAA GCT TT(A/G/T) (C/T) (A/T) (A/T) (A/C/T)AA (C/T)GA (C/T)TA (C/T)AA	
10	<u>ZC3227:</u> CAC CGG CAC CAT CGA T(G/A/)T C(A/C/G/T)A (G/A) (C/T)T C(A/G/C/T)G T(A/G/T)A T	

The PCR reactions were performed for 40 cycles as follows:

15	94 °C	1.5 min
	50 °C	2.0 min
	72 °C	2.0 min

The 180 bp band was eluted from an agarose gel fragment,
20 digested with the restriction enzymes Hind III and Cla I and
ligated into pUC19 which had been digested with Hind III and
AccI. The ligated DNA was transformed into E. coli and mini-
prep DNA was prepared from colony isolates. The DNA sequence of
the cloned DNA was determined. This fragment encoded sequences
25 corresponding to a new member of the F-family cellulases.

Construction of a Fusarium oxysporum cDNA library

Fusarium oxysporum was grown by fermentation and samples were
30 withdrawn at various times for RNA extraction and cellulase
activity analysis. The activity analysis included an assay for
total cellulase activity as well as one for colour
clarification. Fusarium oxysporum samples demonstrating maximal
colour clarification were extracted for total RNA from which
35 poly(A)+RNA was isolated.

To construct a Fusarium oxysporum cDNA library, first-strand cDNA was synthesized in two reactions, one with and the other without radiolabelled dATP. A 2.5X reaction mixture was prepared at room temperature by mixing the following reagents 5 in the following order: 10 μ l of 5X reverse transcriptase buffer (Gibco-BRL, Gaithersburg, Maryland) 2.5 μ l 200 mM dithiothreitol (made fresh or from a stock solution stored at -70°C), and 2.5 μ l of a mixture containing 10 mM of each deoxynucleotide triphosphate, (dATP, dGTP, dTTP and 5-methyl 10 dCTP, obtained from Pharmacia LKB Biotechnology, Alameda, CA). The reaction mixture was divided into each of two tubes of 7.5 μ l. 1.3 μ l of 10 μ Ci/ μ l 32 P α -dATP (Amersham, Arlington Heights, IL) was added to one tube and 1.3 μ l of water to the other. Seven microliters of each mixture was transferred to 15 final reaction tubes. In a separate tube, 5 μ g of Fusarium oxysporum poly (A)⁺ RNA in 14 μ l of 5 mM Tris-HCl pH 7.4, .50 μ M EDTA was mixed with 2 μ l of 1 μ g/ μ l first strand primer (ZC2938 GACAGAGCACAGAATTCACTAGTGAGCTCT₁₅). The RNA-primer mixture was heated at 65°C for 4 minutes, chilled in ice water, and 20 centrifuged briefly in a microfuge. Eight microliters of the RNA-primer mixture was added to the final reaction tubes. Five microliters of 200 U/ μ l SuperscriptTM reverse transcriptase (Gibco-BRL) was added to each tube. After gentle agitation, the tubes were incubated at 45°C for 30 minutes. Eighty microliters 25 of 10 mM Tris-HCl pH 7.4, 1 mM EDTA was added to each tube, the samples were vortexed, and briefly centrifuged. Three microliters was removed from each tube to determine counts incorporated by TCA precipitation and the total counts in the reaction. A 2 μ l sample from each tube was analyzed by gel 30 electrophoresis. The remainder of each sample was ethanol precipitated in the presence of oyster glycogen. The nucleic acids were pelleted by centrifugation, and the pellets were washed with 80% ethanol. Following the ethanol wash, the samples were air dried for 10 minutes. The first strand 35 synthesis yielded 1.6 μ g of Fusarium oxysporum cDNA, a 33% conversion of poly(A)+RNA into DNA.

Second strand cDNA synthesis was performed on the RNA-DNA hybrid from the first strand reactions under conditions which encouraged first strand priming of second strand synthesis resulting in hairpin DNA. The first strand products from each 5 of the two first strand reactions were resuspended in 71 μ l of water. The following reagents were added, at room temperature, to the reaction tubes: 20 μ l of 5X second strand buffer (100 mM Tris pH 7.4, 450 mM KCl, 23 mM MgCl₂, and 50 mM (NH₄)₂(SO₄), 3 μ l of 5 mM β -NAD, and μ l of a deoxynucleotide triphosphate 10 mixture with each at 10 mM. One microliter of α -³²P dATP was added to the reaction mixture which received unlabeled dATP for the first strand synthesis while the tube which received labeled dATP for first strand synthesis received 1 μ l of water. Each tube then received 0.6 μ l of 7 U/ μ l E. coli DNA ligase 15 (Boehringer-Mannheim, Indianapolis, IN), 3.1 μ l of 8 U/ μ l E. coli DNA polymerase I (Amersham), and 1 μ l 2 U/ μ l of RNase H (Gibco-BRL). The reactions were incubated at 16°C for 2 hours. After incubation, 2 μ l from each reaction was used to determine TCA precipitable counts and total counts in the reaction, and 20 2 μ l from each reaction was analyzed by gel electrophoresis. To the remainder of each sample, 2 μ l of 2.5 μ g/ μ l oyster glycogen, 5 μ l of 0.5 EDTA and 200 μ l of 10 mM Tris-HCl pH 7.4, 1 mM EDTA were added. The samples were phenol-chloroform extracted and isopropanol precipitated. After centrifugation 25 the pellets were washed with 100 μ l of 80% ethanol and air dried. The yield of double stranded cDNA in each of the reactions was approximately 2.5 μ g.

Mung bean nuclease treatment was used to clip the single-stranded DNA of the hair-pin. Each cDNA pellet was resuspended in 15 μ l of water and 2.5 μ l of 10X mung bean buffer (0.3 M NaAc pH 4.6, 3 M NaCl, and 10 mM ZnSO₄), 2.5 μ l of 10 mM DTT, 2.5 μ l of 50% glycerol, and 2.5 μ l of 10 U/ μ l mung bean nuclease (New England Biolabs, Beverly, MA) were added to each 30 tube. The reactions were incubated at 30°C for 30 minutes and 75 μ l of 10 mM Tris-HCl pH 7.4 and 1 mM EDTA was added to each tube. Two-microliter aliquots were analyzed by alkaline agarose 35

gel analysis. One hundred microliters of 1 M Tris-HCl pH 7.4 was added to each tube and the samples were phenol-chloroform extracted twice. The DNA was isopropanol precipitated and pelleted by centrifugation. After centrifugation, the DNA 5 pellet was washed with 80% ethanol and air dried. The yield was approximately 2 μ g of DNA from each of the two reactions.

The cDNA ends were blunted by treatment with T4 DNA polymerase. DNA from the two samples were combined after resuspension in a 10 total volume of 24 μ l of water. Four microliters of 10X T4 buffer (330 mM Tris-acetate pH 7.9, 670 mM KAc, 100 mM MgAc, and 1 mg/ml gelatin), 4 μ l of 1 mM dNTP, 4 μ l 50 mM DTT, and 4 μ l of 1 U/ μ l T4 DNA polymerase (Boehringer-Mannheim) were added to the DNA. The samples were incubated at 15°C for 1 hour. 15 After incubation, 160 μ l of 10 mM Tris-HCl pH 7.4, 1 mM EDTA was added, and the sample was phenol-chloroform extracted. The DNA was isopropanol precipitated and pelleted by centrifugation. After centrifugation the DNA was washed with 80% ethanol and air dried.

20

After resuspension of the DNA in 6.5 μ l water, Eco RI adapters were added to the blunted DNA. One microliter of 1 μ g/ μ l Eco RI adapter (Invitrogen, San Diego, CA Cat. # N409-20), 1 μ l of 10X ligase buffer (0.5 M Tris pH 7.8 and 50 mM MgCl₂), 0.5 μ l of 10 25 mM ATP, 0.5 μ l of 100 mM DTT, and 1 μ l of 1 U/ μ l T4 DNA ligase (Boehringer-Mannheim) were added to the DNA. After the sample was incubated overnight at room temperature, the ligase was heat denatured at 65°C for 15 minutes.

30 The Sst I cloning site encoded by the first strand primer was exposed by digestion with Sst I endonuclease. Thirty-three microliters of water, 5 μ l of 10X Sst I buffer (0.5 M Tris pH 8.0, 0.1 M MgCl₂, and 0.5 M NaCl), and 2 μ l of 5 U/ μ l Sst I were added to the DNA, and the samples were incubated at 37°C 35 for 2 hours. One hundred and fifty microliters of 10 mM Tris-HCl pH 7.4, 1 mM EDTA was added, the sample was phenol-chloroform extracted, and the DNA was isopropanol precipitated.

The cDNA was chromatographed on a Sepharose CL 2B (Pharmacia LKB Biotechnology) column to size-select the cDNA and to remove free adapters. A 1.1 ml column of Sepharose CL 2B was poured into a 1 ml plastic disposable pipet and the column was washed 5 with 50 column volumes of buffer (10 mM Tris pH 7.4 and 1 mM EDTA). The sample was applied, one-drop fractions were collected, and the DNA in the void volume was pooled. The fractionated DNA was isopropanol precipitated. After centrifugation the DNA was washed with 80% ethanol and air 10 dried.

A Fusarium oxysporum cDNA library was established by ligating the cDNA to the vector pYcDE8' (cf. WO 90/10698) which had been digested with Eco RI and Sst I. Three hundred and ninety 15 nanograms of vector was ligated to 400 ng of cDNA in a 80 μ l ligation reaction containing 8 μ l of 10 X ligase buffer, 4 μ l of 10 mM ATP, 4 μ l 200 mM DTT, and 1 unit of T4 DNA ligase (Boehringer-Mannheim. After overnight incubation at room temperature, 5 μ g of oyster glycogen and 120 μ l of 10 mM Tris- 20 HCl and 1 mM EDTA were added and the sample was phenol-chloroform extracted. The DNA was ethanol precipitated, centrifuged, and the DNA pellet washed with 80% ethanol. After air drying, the DNA was resuspended in 3 μ l of water. Thirty seven microliters of electroporation competent DH10B cells 25 (Gibco-BRL) was added to the DNA, and electroporation was completed with a Bio-Rad Gene Pulser (Model #1652076) and Bio-Rad Pulse Controller (Model #1652098) electroporation unit (Bio-Rad Laboratories, Richmond, CA). Four milliliters of SOC (Hanahan, J. Mol. Biol. 166 (1983), 557-580) was added to the 30 electroporated cells, and 400 μ l of the cell suspension was spread on each of ten 150 mm LB ampicillin plates. After an overnight incubation, 10 ml of LB amp media was added to each plate, and the cells were scraped into the media. Clycerol stocks and plasmid preparations were made from each plate. The 35 library background (vector without insert) was established at approximately 1% by ligating the vector without insert and titrating the number of clones after electroporation.

Screening the cDNA library

Full length cellulase cDNA clones were isolated from the Fusarium oxysporum cDNA library by hybridization to PCR 5 generated genomic oligonucleotide probes.

The PCR-generated oligonucleotides: ZC3309, a 40-mer coding for part of the C family cellobiohydrolase, ATT ACC AAC ACC AGC GTT GAC ATC ACT GTC AGA GGG CTT C; ZC3310, a 28-mer coding for the 10 C family endoglucanase, AAC TCC GTT GAT GAA AGG AGT GAC GTA G; and ZC3311, a 40-mer coding for the F family cellulase, CGG AGA GCA GCA GGA ACA CCA GAG GCA GGG TTC CAG CCA C, were end labeled with T₄ polynucleotide kinase and ³²P gamma ATP. For the kinase reaction 17 picomoles of each oligonucleotide were 15 brought up to 12.5 μ l volume with deionized water. To these were added 2 μ l 10 X kinase buffer (1 X: 10 mM magnesium chloride, 0.1 mM EDTA, 50 mM Tris pH 7.8), 0.5 μ l 200 mM dithiothreitol, 1 μ l ³²P gamma ATP 150 mCi/ml, Amersham), 2 μ l 20 mixed and incubated at 37°C for 30 minutes. Oligonucleotides 20 were separated from unincorporated nucleotides by precipitation with 180 μ l TE (10 mM tris pH 8., 1 mM EDTA), 100 μ l 7.5 M ammonium acetate, 2 μ l mussel glycogen (20 mg/ml, Gibco-BRL) and 750 μ l 100% ethanol. Pellets were dissolved in 200 μ l 25 distilled water. To determine the amount of radioactivity incorporated in the oligonucleotides, 10 μ l of 1:1000 dilutions of oligonucleotides were read without scintillation fluid in a Beckman LS 1800 Liquid Scintillation System. Activities were: 30 115 million cpm for ZC3309, 86 million cpm for ZC3310, and 79 million cpm for ZC3311.

Initially, a library of 20,000 cDNA clones was probed with a mixture of each of the three oligonucleotides corresponding to the C family cellobiohydrolase, C family endoglucanase and F 35 family cellulase clones. The cDNA library was plated out from titered glycerol stocks stored at -70°C. Four thousand clones were plated out on each of five 150 mm LB ampicillin (1000

$\mu\text{g}/\text{ml}$) plates. Lifts were taken in duplicate following standard methodology (Sambrook et al., Molecular Cloning, 1989) using Biotrans 0.2 μm 137 mm filters. The filters were baked at 80°C in vacuum for 2 hours, then swirled overnight in a 5 crystallizing dish (Pharmacia LKB Biotechnology, Alameda, CA) at 37°C in 80 ml prehybridization solution (5 X Denhardt's (1X: 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumen Pentax Fraction 5 (Sigma, St. Louis, MO)) 5 X SSC (1 X: 0.15 M sodium chloride, 0.15 M sodium citrate pH 7.3)), 100 10 $\mu\text{g}/\text{ml}$ denatured sonicated salmon sperm DNA, 50 mM sodium phosphate pH 6.8, 1 mM sodium pyrophosphate, 100 μM ATP, 20% formamide, 1% sodium dodecyl sulfate) (Ulrich et al. EMBO J. 3 (1984), 361-364).

15 Prehybridized filters were probed by adding them one at a time into a crystallizing dish with 80 ml prehybridization solution with 80 million cpm ZC3309, 86 million cpm ZC3310 and 79 million cpm ZC3311 and then swirled overnight at 37°C. Filters were then washed to high stringency. The probed filters were 20 washed with three 400 ml volumes of low stringency wash solution (2 X SSC, 0.1% SDS) at room temperature in the crystallizing dish, then with four 1-liter volumes in a plastic box. A further wash for 20 minutes at 68°C with tetramethylammonium chloride wash solution (TMACL: 3 M 25 tetramethylammonium chloride, 50 mM Tris-HCl pH 8.0, 2 mM EDTA, 1 g/l SDS) (Wood et al., Proc. Natl. Acad. Sci. 82 (1985)) provided a high stringency wash for the 28-mer ZC3310 independent of its base composition 1585-1588). The filters were then blotted dry, mounted on Whatman 3MM paper and covered 30 with plastic wrap for autoradiography. They were exposed overnight at -70°C with intensifying screens and Kodak XAR-5 film.

Two putative positives appeared on duplicate filters. The 35 corresponding areas on the plates with colonies were picked into 1 ml of 1X polymerase chain reaction (PCR) buffer (100 mM Tris HCl pH 8.3, 500 mM KCl, 15 mM MgCl₂, 0.1% gelatin; Perkin

Elmer Cetus) and plated out at five tenfold dilutions on 100 mm LB plates with 70 µg/ml ampicillin. These plates were grown at 37°C overnight. Two dilutions of each putative clone were chosen for rescreening as outlined above. One isolated clone,
5 pZFH196 was found. This was grown up overnight in 10 ml 2X YT broth (per liter: 16 g bacto-tryptone, 10 g bacto-yeast extract, 10 g NaCl). Twenty three micrograms of DNA were purified by the rapid boiling method (Holmes and Quigley, Anal. Biochem. 114 (1981), 193-197). From restriction analysis
10 the clone was found to be approximately 2,000 base pairs in length. Sequence analysis showed it to contain a fragment homologous to the C family cellobiohydrolase fragment cloned by PCR.

15 In an attempt to isolate additional cellulase cDNA clones, a cDNA library of 2 million clones was plated out on 20 150 mm LB plates (100 µg/ml ampicillin) containing approximately 100,000 cDNA clones. Lifts were taken in duplicate as in the first screening attempt. This library was screened with
20 oligonucleotides corresponding to the three cellulase species as described above except that the hybridization was carried out with formamide in the prehybridization buffer and at a temperature of 30°C. Washing with TMACL was carried out twice for 20 minutes at 67°C. Between 8 and 20 signals were found on
25 duplicate filters of each of the 20 plates. Fifteen plugs were taken from the first plate with the large end of a pasteur pipet into 1 ml 1 X PCR buffer (Perkin-Elmer Cetus). PCR was carried out on the bacterial plugs with three separate oligonucleotide mixtures. Each mixture contained the vector
30 specific oligonucleotide ZC2847 and additionally, a different cellulase specific oligonucleotide (ZC3309, ZC3310 or ZC3311) within each mixture. AmpliTaq polymerase (Perkin-Elmer Cetus) was used with Pharmacia Ultrapure dNTP and following Perkin Elmer Cetus procedures. Sixteen picomoles of each primer were
35 used in 40 µl reaction volumes. Twenty microliters of cells in 1 X PCR buffer were added to 20 µl mastermix which contained everything needed for PCR except for DNA. After an initial 1

minute 45 second denaturation at 94°C 28 cycles of: 45 seconds at 94°C, 1 minute at 45°C and 2 minutes at 72°C with a final extension of 10 minutes at 72°C were employed in a Perkin Elmer thermocycler. Ten of the 15 plugs yielded a band when primed 5 with the C family specific oligonucleotide ZC3309 and ZC2847. The other mixtures gave no specific products. Five plugs which produced the largest bands by PCR, therefore possibly being full length C family cellobiohydrolases, along with the 5 plugs which did not produce PCR bands, were plated out at five 10 10 fold dilutions onto 100 mm LB plates with 70 µg/ml ampicillin and grown overnight. Duplicate lifts were taken of two ten fold dilutions each. Prehybridization and hybridization were carried out as described above with a mixture of the 3 oligonucleotides. Isolated clones were found on all 10 of the 15 platings. These were picked from the dilution plates with a toothpick for single colony isolation on 100 mm LB plates with 70 µg/ml ampicillin. PCR was carried out on isolated bacterial colonies with 2 oligonucleotides specific for the C family cellobiohydrolase (ZC3409 (CCG TTC TGG ACG TAC AGA) and ZC3411 20 (TGA TGT CAA GTT CAT CAA)). Conditions were identical to those described above except for using 10 picomoles of each primer in 25 µl reaction volumes. Colonies were added by toothpick into PCR tubes with 25 µl mastermix before cycling. Five of the 10 gave strong bands of the size expected for a C family 25 cellobiohydrolase. Isolated colonies were then grown up in 20 ml of Terrific Broth (Sambrook et al., op. cit., A2) and DNA was isolated by the rapid boiling method. The clones were partially sequenced by Sanger dideoxy sequencing. From sequence analysis the 5 clones which did not give bands specific for a 30 C family cellobiohydrolase by PCR were shown to be F family cellulase clones.

In order to clone the C family endoglucanase, the cDNA library of 2 million clones was rescreened with only ZC3310. Conditions 35 of prehybridization and hybridization were like those used above. Filters were hybridized for 10 hours at 30°C with one million CPM endlabeled ZC3310 per ml prehybridization solution

without formamide. Washing with TMACl was carried out 2 times for 20 minutes at 60°C. Seven weak signals were found on duplicate filters. Plugs were picked with the large end of a pipet into 1 ml LB broth. These were each plated out in 5 10 fold dilutions on 100 mm LB plates with 70 µg/ml ampicillin. Duplicate lifts were taken of 2 dilutions each and were processed as described above. Prehybridization, hybridization, and washing were carried out as for the first level of screening. Three isolated clones were identified and streaked out for single colony hybridization. Isolates were grown overnight in 50 ml of Terrific Broth (per liter: 12 g tryptone, 24 g yeast extract, 4 ml glycerol, autoclaved, and 100 ml of 0.17 M KH₂PO₄, 0.72 M K₂HPO₄ (Sambrook et al., op. cit., A2) and DNA was isolated by alkaline lysis and PEG precipitation by standard methods (Maniatis 1989, 1.38-1.41). From restriction analysis, one clone (pZFH223) was longer than the others and was chosen for complete sequencing. Sequence analysis showed it to contain the PCR fragment cloned initially.

20 DNA sequence analysis

The cDNAs were sequenced in the yeast expression vector pYCDE8'. The dideoxy chain termination method (F. Sanger et al., Proc. Natl. Acad. Sci. USA 74, 1977, pp. 5463-5467) using α 35-S dATP from New England Nuclear (cf. M.D. Biggin et al., Proc. Natl. Acad. Sci. USA 80, 1983, pp. 3963-3965) was used for all sequencing reactions. The reactions were catalysed by modified t7 DNA polymerase from Pharmacia (cf. S. Tabor and C.C. Richardson, Proc. Natl. Acad. Sci. USA 84, 1987, pp. 4767-4771) and were primed with an oligonucleotide complementary to the ADH1 promoter (ZC996: ATT GTT CTC GTT CCC TTT CTT), complementary to the CYC1 terminator (ZC3635: TGT ACG CAT GTA ACA TTA) or with oligonucleotides complementary to the DNA of interest. Double stranded templates were denatured with NaOH (E.Y. Chen and P.H. Seeburg, DNA 4, 1985, pp. 165-170) prior to hybridizing with a sequencing oligonucleotide. Oligonucleotides were synthesized on an Applied Biosystems Model 380A DNA

synthesizer. The oligonucleotides used for the sequencing reactions are listed in the sequencing oligonucleotide table below:

5 C-family cellobiohydrolase sequencing primers

ZC3411 TGA TGT CAA GTT CAT CAA
ZC3408 TCT GTA CGT CCA GAA CGG
ZC3407 ATG ACT TCT CTA AGA AGG
ZC3406 TCC AAC ATC AAG TTC GGT
10 ZC3410 AGG CCA ACT CCA TCT GAA
ZC3309 ATT ACC AAC ACC AGC GTT GAC ATC ACT GTC AGA GGG CTC
C
ZC3409 CCG TTC TGG ACG TAC AGA

15 F-family cellulase specific sequencing primers

ZC3413 CCA TCG ACG GTA TTG GAT
ZC3311 CGG AGA GCA GCA GGA ACA CCA GAG GCA GGG TTC CAG CCA
C
ZC3412 GAG GGT AGA GCG ATC GTT

20

C-family endoglucanase specific sequencing primers

ZC3739 TGA TCT CAT CGA GCT GCA CC
ZC3684 GTG ATG CTC AGT GCT ACG TC
ZC3310 AAC TCC GTT GAT GAA AGG AGT GAC GTA G
25 ZC3750 TCC AAT AGC TTC CCA GCA AG
ZC3683 TGT CCC TTG ATG TTG CCA AC

The DNA sequences of the full-length cDNA clones, as well as the derived amino acid sequences, are shown in the appended
30 Figs. 11 (C-family cellobiohydrolase), 12 (F-family cellulase) and 13 (C-family endoglucanase).

Example 4Isolation of endoglucanase EGI gene from H. insolens

The cDNA library described in example 1 was also screened with
5 a 35 bp oligonucleotide probe in the antisense configuration
with the sequence:

NOR-770: 5' GCTTCGCCCATGCCTGGGTGGCGCCGAGTTCCAT 3'

The sequence was derived from the amino acid sequence of an
10 alcalase fragment of EGI purified from H. insolens, using our
knowledge of codon bias in this organism. Complete clones of
1.6 kb contained the entire coding sequence of 1.3 kb as shown
in Fig. 14A-E. The probe sequence NOR-770 is located at Met₃₄₄-
Ala₃₅₅.

15

Construction of expression plasmids of EGI (full length) and
EGI' (truncated)

The EGI gene still containing the poly-A tail was inserted into
20 an A. oryzae expression plasmid as outlined in Fig 2. The
coding region of EGI was cut out from the NcoI-site in the
initiating Met-codon to the Bam H1-site downstream of the poly-
A region as a 1450 bp fragment from pHW480. This was ligated to
a 3.6 kb NcoI-NarI fragment from pSX224 (Fig. 1) containing the
25 TAKA promoter and most of pUC19, and to a 960 bp NarI-BamHI
fragment containing the remaining part with the AMG-terminator.
The 960 bp fragment was taken from p960 which is equivalent to
p777 (described in EP 238,023) except for the inserted gene.
The resulting expression plasmid is termed pHW485.

30

The expression plasmid pHW704 with the full length EGI gene
without poly A tail is shown in Fig. 3. From the BstEII site
1300 bp downstream of the NcoI-site was inserted a 102 bp
BstEII-BamHI linker (2645/2646) ligated to BglIII-site in the
35 vector. The linker contains the coding region downstream of
BstEII-site with 2 stop codons at the end and a PvuI-site near
the C-terminal to be used for addition of CBD and B-regions.

Expression plasmid pHW697 with the truncated EGI' gene was constructed similarly using a BstEII-BamHI linker (2492/2493) of 69 bp. In this linker we introduced a PstI-site altering Val₄₂₁ to Leu₄₂₁ and the last 13 amino acids of the coding 5 region: K₄₂₃PKPKPGHGPRSD₄₃₅ were eliminated. The short tail with the rather unusual sequence was cut off to give EGI' a C-terminal corresponding to the one found in T. reesei EGI just upstream of the A and B-region.

10

Construction of an expression plasmid of EGI' with CBD and B region from a ~ 43 kD endoglucanase added C-terminally

The ~ 43 kD endoglucanase of H. isolens described in DK patent 15 application No. 736/91 has shown good washing performance. Besides the catalytic domain, 43 kD cellulase has a C-terminal CBD and B region which has been transferred to EGI' which does not have any CBD or B region itself. The construction was done in 2 steps, as outlined in Fig. 4. The PstI-HincII linker 20 (028/030 M) intended to connect the C-terminal of EGI' to the B-region of 43 kD cellulase, was subcloned in pUC19 PstI-EcoRI with C-terminal Hinc2-EcoRI 100 bp fragment from 43 kD cellulase gene in pSX320 (Fig 5; as described in DK 736/91). From the subclone pHW767 the CBD and B-region was cut out as a 25 250 bp PstI-BglII fragment and ligated to pHW485 (Fig. 2) BstEII-BglII fragment of 5.7 kb and to the remaining BstEII-PstI fragment of 55 bp from pHW697 (Fig. 3). The resulting expression plasmid pHW768 has the ~ 43 kD endoglucanase CBD and B region added to Gln₄₂₂ of EGI'.

30

Construction of an expression plasmid of EGI with the CBD and B region from ~ 43 kD endoglucanase added C-terminally

35 This plasmid was constructed in a similar way as pHW768 except that, in this case, the C-terminal linker yielded the complete sequence of EGI. Fig. 6 shows the procedure in 3 steps. The

PvuI-HincII linker (040 M/041 M) was subcloned in pUC18 to give pHW775, into which a HincII-EcoRI 1000 bp fragment from pSX 320 (Fig. 5) was inserted to give pHW776. From this the CBD and B region was cut out as a 250 bp PvuI-BglII fragment and ligated 5 to 5.7 kb BstEII-BglII fragment from pHW485 (Fig. 2) and 90 bp BstEII-PvuI fragment from pHW704 (Fig. 3). The resulting expression plasmid pHW777 contains the ~ 43 kD endoglucanase CBD and B region added to Asp₄₃₅ in the complete EGI sequence.

10

Expression in A. oryzae of EGI and EGI' with and without the CBD and B region from ~ 43 kD endoglucanase

The expression plasmids pHW485, pHW704, pHW697, pHW768 and 15 pHW777 were transformed into A. oryzae IFO 4177 as described in example 2. Supernatants from transformants grown in YPD medium as described were analyzed by SDS-PAGE, where the native EGI has an apparent Mw of 53 kD. EGI' looks slightly smaller as expected, and the species with the added CBD and B region are 20 increased in molecular weight corresponding to the size of the CBD and B region with some carbohydrate added. A polyclonal antibody AS169 raised against the ~ 43 kD endoglucanase recognizes EGI and EGI' only when the ~ 43 kD CBD and B region are added, while all 4 species are recognized by a polyclonal 25 antibody AS78 raised against a cellulase preparation from H. insolens. All 4 species have endoglucanase activity as measured on soluble cellulose in the form of carboxy methyl cellulose.

Linkers

30

2492/2493:

BstE2-PstI-BamH1

5' GTCACCTACACCAACCTCCGCTGGGGCGAG
3' GATGTGGTTGGAGGCGACCCCGCTC

35

ATCGGCTCGACCTACCAGGAGCTGCAGTAGTAA
TAGCCGAGCTGGATGGTCCTCGACGTCATCATT

40	TGATAG	3'	69 bp
	ACTATCCTAG	5'	68 bp

2645/2646: BstE2-XmaI-PvuI-BamH1

5'	GTCACCTACACCAACCTCCGCTGGGGCGAGATCGGC	
3'	GATGTGGTTGGAGGCAGCCCCGCTCTAGCCG	
5	TCGACCTACCAGGAGGTTCAGAAGCTAACGCCAAG	
	AGCTGGATGGTCTCCAAGTCTTCGGATTGGTTTC	
10	CCCAGGCACGGCCCCCGATCGGACTAAATAG	3' 102 bp
	GGGCGCGTGCCGGGGCTAGCCTGATTATCCTAG	5' 101 bp

028 M/030 M: PstI-HincII

15 5'	GTCCAGCAGCACCAAGCTCTCCGGTC	3' 25 bp
3'	ACGTCAGGTCGTCGTGGTCGAGAGGCCAG	5' 29 bp

040 M/041 M: PvuI-HincII

20 5'	CGTCCAGCAGCACCAAGCTCTCCGGTC	3' 26 bp
3'	TAGCAGGTCGTCGTGGTCGAGAGGCCAG	5' 28 bp

25 Example 5**- 43 kD endoglucanase with different CBDs and B-regions:**

In order to test the influence on the - 43 kD endoglucanase of
 30 the different CBDs and B regions from the A region clones we
 have substituted the original CBD and B region from ~ 43 kD
 with the other C-terminal CBDs and B regions, i.e. A-1, A-8, A-
 9, A-11, and A-19 (cf. Example 1). In order to test the
 concept we have also made a construction where the 43 kD B
 35 region has been deleted.

Fragments:

40 All fragments were made by PCR amplification using a Perkin-
 Elmer/Cetus DNA Amplification System following the
 manufacturers instructions.

1) A PCR fragment was made which covers the DNA from 56 bp upstream of the Bam HI site on pSX 320 (Fig. 5) to 717 bp within the coding region of the ~43 kD endoglucanase gene and at the same time introduces a Kpn I site at pos. 708 and a Sma I site at pos. 702 in the coding region which is at the very beginning of the B region. This PCR fragment was made with the primers NOR 1542 and NOR 3010 (see list of oligonucleotides below).

10

2) A PCR fragment was made which includes the CBD and B region of A-1 introducing a Kpn I site at the very beginning of the B region in frame with the Kpn I site introduced in 1) and introducing a Xho I site downstream of the coding region of the gene. Primers used: NOR 3012 upstream and NOR 3011 downstream.

3) As 2) except that the fragment covered the CBD and B region of A-8 and the Xho I site in the expression vector downstream of gene. Primers: NOR 3017 and NOR 2516.

20

4) As 2) but with primers NOR 3016 and NOR 3015 covering the CBD and B region from A-9.

5) As 3) but with primers NOR 3021 and NOR 2516 covering the CBD and B region from A-11.

6) As 2) but with primers NOR 3032 and NOR 3022 covering the CBD and B region from A-19.

30 7) A PCR fragment which includes the CBD from ~ 43 kD endoglucanase and the Xho I site downstream from the gene on pSX 320 introducing a Pvu II site at the very end of the B region.

Primers: NOR3023 and NOR2516.

35

Combinations:

1) + 2) inserted as Bam HI - Kpn I and Kpn I - Xho I into pToC
68 (described in DK736/91) Bam HI - Xho I, thus coding for the
43 kD core enzyme with the CBD and B region from A-1.

5 1) + 3): Like above giving a 43 kD enzyme with the A-8 CBD/B
region.

1) + 4): As above, but with the A-9 CBD and B region.

10 1) + 5): As above, but with the A-11 CBD and B region.

1) + 6): As above, but with the A-19 CBD and B region.

15 1) + 7) inserted as Bam HI - Sma I and Pvu II - Xho I into pToC
68 Bam HI - Xho I, thus coding for the 43 kD enzyme without the
B region.

Oligonucleotides:

20

NOR 1542: 5' - CGACAAACATCACATCAAGCTCTCC - 3'

NOR 2516: 5' - CCATCCTTTAACTATAGCGA - 3'

25

NOR 3010: 5' - GCTGGTGCTGGTACCCGGGATCTGGACGGCAGGG - 3'
Kpn Sma

NOR 3011: 5' - GCATCGGTACCGGGCGGCCACTGGCG - 3'
Kpn

30

NOR 3012: 5' - CTCACTCCATCTCGAGTCTTTCAATTACA - 3'
Xho

35

NOR 3015: 5' - CTTTCTCGAGTCCCTTAGTTCAAGCACTGC - 3'
Xho

NOR 3016: 5' - TGACCCGGTACCGGGCGGCCAACACCAACC - 3'
Kpn

40

NOR 3017: 5' - TCACCCGGTACCGGGCGGTGGAAGCAACAATG - 3'
Kpn

NOR 3021: 5' - TCTTCGGTACCAAGCGGCAACAGCGGGCGCG - 3'
Kpn

45

37

NOR 3022: 5' - CGCTGGGTACCAACAACAATCCTCAGCAGG - 3'
 Kpn

NOR 3023: 5' - CTCCCAGCAGCTGCACTGCTGAGAGGTGGG - 3'
 5 Pvu II

NOR 3032: 5' - CGGCCTCGAGACCTTACAGGCACTGCGACT - 3'
 Xho

10

Example 6

Fusion of a bacterial catalytic domain to a fungal CBD

15 The endoglucanase Endo 1 produced by Bacillus lautus NCIMB 40250 (described in PCT/DK91/00013) consists of a catalytic domain (core) (Ala(32) - Val(555)) and a C terminal cellulose binding domain (CBD) (Gln556 - Pro700) homologous to the CBD of a B. subtilis endoglucanase (R.M. MacKay et al. 1986. Nucleic 20 Acids Res. 14, 9159-70). The CBD is proteolytically cleaved off when the enzyme is expressed in B. subtilis or E. coli generating a CMC degrading core enzyme. In this example this core protein was fused with the B region and CBD of the ~ 43 kD endoglucanase from Humicola insolens (described in DK 736/91).

25

Construction of the fusion.

The plasmid pCaHj 170 containing the cDNA gene encoding the ~ 43 kD endoglucanase was constructed as shown in Fig. 7. pCaHj 30 170 was digested with Xho II and Sal I. The 223 bp Xho II - Sal I fragment was isolated and ligated into pUC 19 (Yanisch-Perron et al. 1985. Gene 33, 103-119) digested with BamH I and Sal I. The BamH I site was regenerated by this Xho II-BamH I ligation. The resulting plasmid, IM 2, was digested with Eco R1 and BamH 35 I and ligated with the linker NOR 3045 - NOR 3046:

NOR 3045	5'	AATTCCGGGAACGATATCTCCGA	3'
NOR 3046	3'	GGCGCCTTGCTATAGAGGCTCTAG	5'
		EcoR I EcoR V Mbo I	
40		Sac II	

The resulting plasmid, IM 3, was digested with EcoR V and SacII and ligated to the 445 bp Hinc II - Sac II pPL 517 fragment. pPL 517 contains the entire Bacillus Endo 1 gene (PCT/DK91/00013). The product of this ligation was termed IM 4.

5 In order to replace the Bacillus signal peptide of Endo 1 with the fungal signal peptide from the 43 kdal endoglucanase four PCR primers were designed for "Splicing by Overlap Extension" (SOE) fusion (R M Horton et al.(1989):Gene, 77, 61-68). The 43 KD signal sequence was amplified from the plasmid pCaHj 109 (DK 10 736/91) introducing a Bcl I site in the 5'end and a 21 bp homology to the Bacillus endo 1 gene in the 3' end using the 5' primer NOR 3270 and the 3' primer NOR 3275. The part of the Endo I gene 5' to the unique Sac II site was amplified using the 5' primer NOR 3276 introducing a 21 bp homology to the 43 15 kdal gene and the 3' primer NOR 3271 covering the Sac II site. The two PCR framents were mixed, melted, annealed and filled up with the taq polymerase (Fig. 9). The resulting hybrid was amplified using the primers NOR 3270 and NOR 3271. The hybrid fragment was digested with Bcl I and SacII and ligated to the 20 676 bp Sac II - Sal I fragment from IM 4 and the Aspergillus expression vector pToC 68 (DK 736/91) digested with BamH I. The product of this ligation, pCaHj 180 (Fig. 10), contained an open reading frame encoding the 43 kD signal peptide and the first four N terminal aminoacids of the mature ~ 43 kD 25 endoglucanase (Met(1)-Arg(25) fused to the core of Endo 1 (Ser(34)-Val(549)) followed by the peptide Ile-Ser-Glu (encoded by the linker) fused to the 43 kD B region and CBD (Ile(233)-Leu(285)). pCaHj 180 was used to transform Aspergillus oryzae IFO 4177 using selection on acetamide by cotransformation with 30 pToC 90 (cf. DK 736/91) as described in published EP patent application No. 238 023.

NOR 3270 5' TTGAATTCTGATCAAGATGCGTTCCTCCC 3'
NOR 3275 5' AATGGTGAAAGTGACATCACTCCTGCCATCAGCGGCAAGGGC 3'
35 NOR 3276 5' GCCCTGCCGCTGATGGCAGGAGTGATGTCACTTCAACCATT 3'
NOR 3271 5' AGCGCGTCCGGTAGCTATG 3'

The sequence of the Endo 1 core and the ~ 43 kD CBD and B region is shown in the appended Fig. 15A-D.

CLAIMS

1. A cellulose- or hemicellulose-degrading enzyme which is
derivable from a fungus other than Trichoderma or
5 Phanerochaete, and which comprises a carbohydrate binding
domain homologous to a terminal A region of Trichoderma reesei
cellulases, which carbohydrate binding domain comprises the
following amino acid sequence

10 1 Xaa Xaa Gln Cys Gly Gly Xaa Xaa Xaa Xaa Gly Xaa Xaa Xaa Cys Xaa
15 20 Xaa Xaa Xaa Xaa Cys Xaa Xaa Xaa Asn Xaa Xaa Tyr Xaa Gln Cys Xaa
Xaa -
15

or a subsequence thereof capable of effecting binding of the
20 enzyme to an insoluble cellulosic or hemicellulosic substrate.

2. An enzyme according to claim 1, which is derivable from a strain of *Humicola*, *Fusarium* or *Myceliothpora*.

25 3. An enzyme according to claim 1, wherein the variations in
the amino acid sequence shown in claim 1 are selected as
follows

in position 1, the amino acid is Trp or Tyr;

in position 2, the amino acid is Gly or Ala;

30 in position 7, the amino acid is Gln, Ile or Asn;

in position 8, the amino acid is Gly or Asn;

in position 9, the amino acid is Trp, Phe or Tyr:

in position 10, the amino acid is Ser, Asn, Thr or

in position 12, the amino acid is Pro, Ala or Cys.

in position 13, the amino acid is Thr, Arg or Lys.

in position 14, the amino acid is Thr, Cys or Asn.

in position 18, the amino acid is Gly or Pro.

In position 11, the amino acid is glycine or proline, in position 12, the amino acid (if present) is

In position 19, the amino acid (if present) is Ser, Thr, Phe, Leu or Ala;

40 in position 20, the amino acid is Thr or Lys:

in position 24, the amino acid is Gln or Ile;
in position 26, the amino acid is Gln, Asp or Ala;
in position 27, the amino acid is Trp, Phe or Tyr;
in position 29, the amino acid is Ser, His or Ala; and/or
5 in position 32, the amino acid is Leu, Ile, Gln, Val or Thr.

4. An enzyme according to claim 3, wherein the carbohydrate binding domain comprises the following amino acid sequence

10 Trp Gly Gln Cys Gly Gly Gln Gly Trp Asn Gly Pro Thr Cys Cys Glu
Ala Gly Thr Thr Cys Arg Gln Gln Asn Gln Trp Tyr Ser Gln Cys
Leu;

Trp Gly Gln Cys Gly Gly Ile Gly Trp Asn Gly Pro Thr Thr Cys Val
15 Ser Gly Ala Thr Cys Thr Lys Ile Asn Asp Trp Tyr His Gln Cys
Leu;

Trp Gly Gln Cys Gly Gly Ile Gly Phe Asn Gly Pro Thr Cys Cys Gln
Ser Gly Ser Thr Cys Val Lys Gln Asn Asp Trp Tyr Ser Gln Cys
20 Leu;

Trp Gly Gln Cys Gly Gly Asn Gly Tyr Ser Gly Pro Thr Thr Cys Ala
Glu Gly - Thr Cys Lys Lys Gln Asn Asp Trp Tyr Ser Gln Cys Thr
Pro;

25 Trp Gly Gln Cys Gly Gly Gln Gly Trp Gln Gly Pro Thr Cys Cys Ser
Gln Gly - Thr Cys Arg Ala Gln Asn Gln Trp Tyr Ser Gln Cys Leu
Asn;

30 Trp Gly Gln Cys Gly Gly Gln Gly Tyr Ser Gly Cys Thr Asn Cys Glu
Ala Gly Ser Thr Cys Arg Gln Gln Asn Ala Tyr Tyr Ser Gln Cys
Ile;

Trp Gly Gln Cys Gly Gly Gln Gly Tyr Ser Gly Cys Arg Asn Cys Glu
Ser Gly Ser Thr Cys Arg Ala Gln Asn Asp Trp Tyr Ser Gln Cys
35 Leu;

Trp Ala Gln Cys Gly Gly Asn Gly Trp Ser Gly Cys Thr Thr Cys Val
Ala Gly Ser Thr Cys Thr Lys Ile Asn Asp Trp Tyr His Gln Cys
Leu;

5 Trp Gly Gln Cys Gly Gly Gln Asn Tyr Ser Gly Pro Thr Thr Cys Lys
Ser Pro Phe Thr Cys Lys Lys Ile Asn Asp Phe Tyr Ser Gln Cys
Gln; or

Trp Gly Gln Cys Gly Gly Asn Gly Trp Thr Gly Ala Thr Thr Cys Ala
10 Ser Gly Leu Lys Cys Glu Lys Ile Asn Asp Trp Tyr Tyr Gln Cys Val

5. An enzyme according to any of claims 1-4, which further
comprises an amino acid sequence which defines a linking B
region connecting the carbohydrate binding domain to the
15 catalytically active domain of the enzyme.

6. An enzyme according to claim 5, wherein the linking B region
is one which is enriched in the amino acids glycine and/or
asparagine and/or proline and/or serine and/or threonine and/or
20 glutamine.

7. An enzyme according to claim 6, wherein one or more of said
amino acids appear in short, repetitive units.

25 8. An enzyme according to any of claims 1-7, which comprises a
carbohydrate binding domain derived from one naturally
occurring cellulose- or hemicellulose-degrading enzyme, an
amino acid sequence defining a linking B region, which amino
acid sequence is derived from another naturally occurring
30 cellulose- or hemicellulose-degrading enzyme, as well as a
catalytically active domain derived from the enzyme supplying
either the carbohydrate binding domain or B region or from a
third enzyme.

35 9. An enzyme according to claim 8, wherein the catalytically
active domain is derived from an enzyme which does not, in
nature, comprise a carbohydrate binding domain or B region.

10. An enzyme according to any of claims 1-9 which is a cellulase, e.g. an endoglucanase, cellobiohydrolase or β -glucosidase.

5 11. A DNA construct which comprises a DNA sequence encoding an enzyme according to any of claims 1-10.

12. An expression vector which carries an inserted DNA construct according to claim 11.

10

13. A cell which is transformed with a DNA construct according to claim 11 or with an expression vector according to claim 12.

14. A cell according to claim 13 which is a fungal cell, e.g. 15 belonging to a strain of Aspergillus, e.g. Aspergillus niger or Aspergillus oryzae, or a yeast cell, e.g. belonging to a strain of Saccharomyces, such as Saccharomyces cerevisiae.

15. A method of producing an enzyme according to any of claims 20 1-10, wherein a cell according to claim 13 or 14 is cultured under conditions conducive to the production of the enzyme, and the enzyme is subsequently recovered from the culture.

16. An agent for degrading cellulose or hemicellulose, the 25 agent comprising an enzyme according to any of claims 1-10.

17. An agent according to claim 16 comprising a combination of two or more enzymes according to any of claims 1-10, or a combination of one or more enzymes according to any of claims 30 1-10 with one or more other enzymes with cellulose- or hemicellulose-degrading activity.

18. A carbohydrate binding domain homologous to a terminal A region of Trichoderma reesei cellulases, which carbohydrate 35 binding domain comprises the following amino acid sequence

1	10	
Xaa Xaa Gln Cys Gly Gly Xaa Xaa Xaa Xaa Gly Xaa Xaa Xaa Cys Xaa		
5	20	30
Xaa Xaa Xaa Xaa Cys Xaa Xaa Xaa Asn Xaa Xaa Tyr Xaa Gln Cys Xaa		
-		
Xaa		

10 or a subsequence thereof capable of effecting binding of a protein to an insoluble cellulosic or hemicellulosic substrate.

19. A carbohydrate binding domain according to claim 18,
wherein the variations in the amino acid sequence shown in
15 claim 18 are selected as follows

in position 1, the amino acid is Trp or Tyr;
in position 2, the amino acid is Gly or Ala;
in position 7, the amino acid is Gln, Ile or Asn;
20 in position 8, the amino acid is Gly or Asn;
in position 9, the amino acid is Trp, Phe or Tyr;
in position 10, the amino acid is Ser, Asn, Thr or Gln;
in position 12, the amino acid is Pro, Ala or Cys;
in position 13, the amino acid is Thr, Arg or Lys;
25 in position 14, the amino acid is Thr, Cys or Asn;
in position 18, the amino acid is Gly or Pro;
in position 19, the amino acid (if present) is Ser, Thr, Phe,
Leu or Ala;
in position 20, the amino acid is Thr or Lys;
30 in position 24, the amino acid is Gln or Ile;
in position 26, the amino acid is Gln, Asp or Ala;
in position 27, the amino acid is Trp, Phe or Tyr;
in position 29, the amino acid is Ser, His or Tyr; and/or
in position 32, the amino acid is Leu, Ile, Gln, Val or Thr.

35

20. A carbohydrate binding domain according to claim 19, wherein the carbohydrate binding domain comprises the following amino acid sequence

Trp Gly Gln Cys Gly Gly Gln Gly Trp Asn Gly Pro Thr Cys Cys Glu
Ala Gly Thr Thr Cys Arg Gln Gln Asn Gln Trp Tyr Ser Gln Cys
Leu;

5 Trp Gly Gln Cys Gly Gly Ile Gly Trp Asn Gly Pro Thr Thr Cys Val
Ser Gly Ala Thr Cys Thr Lys Ile Asn Asp Trp Tyr His Gln Cys
Leu;

Trp Gly Gln Cys Gly Gly Ile Gly Phe Asn Gly Pro Thr Cys Cys Gln
10 Ser Gly Ser Thr Cys Val Lys Gln Asn Asp Trp Tyr Ser Gln Cys
Leu;

Trp Gly Gln Cys Gly Gly Asn Gly Tyr Ser Gly Pro Thr Thr Cys Ala
Glu Gly - Thr Cys Lys Lys Gln Asn Asp Trp Tyr Ser Gln Cys Thr
15 Pro;

Trp Gly Gln Cys Gly Gly Gln Gly Trp Gln Gly Pro Thr Cys Cys Ser
Gln Gly - Thr Cys Arg Ala Gln Asn Gln Trp Tyr Ser Gln Cys Leu
Asn;

20

Trp Gly Gln Cys Gly Gly Gln Gly Tyr Ser Gly Cys Thr Asn Cys Glu
Ala Gly Ser Thr Cys Arg Gln Gln Asn Ala Tyr Tyr Ser Gln Cys
Ile;

Trp Gly Gln Cys Gly Gly Gln Gly Tyr Ser Gly Cys Arg Asn Cys Glu
25 Ser Gly Ser Thr Cys Arg Ala Gln Asn Asp Trp Tyr Ser Gln Cys
Leu;

Trp Ala Gln Cys Gly Gly Asn Gly Trp Ser Gly Cys Thr Thr Cys Val
Ala Gly Ser Thr Cys Thr Lys Ile Asn Asp Trp Tyr His Gln Cys
Leu;

30

Trp Gly Gln Cys Gly Gly Gln Asn Tyr Ser Gly Pro Thr Thr Cys Lys
Ser Pro Phe Thr Cys Lys Lys Ile Asn Asp Phe Tyr Ser Gln Cys
Gln; or

35 Trp Gly Gln Cys Gly Gly Asn Gly Trp Thr Gly Ala Thr Thr Cys Ala
Ser Gly Leu Lys Cys Glu Lys Ile Asn Asp Trp Tyr Tyr Gln Cys Val

21. A linking B region derived from a cellulose- or hemicellulose-degrading enzyme, said region comprising an amino acid sequence enriched in the amino acids glycine and/or asparagine and/or proline and/or serine and/or threonine and/or 5 glutamine.

22. A B region according to claim 21, wherein one or more of said amino acids appear in short, repetitive units.

10 23. A B region according to claim 21 or 22, which comprises the following amino acid sequence

Ala Arg Thr Asn Val Gly Gly Ser Thr Gly Gly Asn Asn Gly
Gly Gly Asn Asn Gly Gly Asn Pro Gly Gly Asn Pro Gly Gly Asn Pro
15 Gly Gly Asn Pro Gly Gly Asn Pro Gly Gly Asn Pro Gly Gly Asn Cys
Ser Pro Leu;

Pro Gly Gly Asn Asn Asn Pro Pro Pro Ala Thr Thr Ser Gln Trp
Thr Pro Pro Pro Ala Gln Thr Ser Ser Asn Pro Pro Pro Thr Gly Gly
20 Gly Gly Gly Asn Thr Leu His Glu Lys;

Gly Gly Ser Asn Asn Gly Gly Asn Asn Asn Gly Gly Asn Asn
Asn Gly Gly Gly Asn Asn Asn Gly Gly Asn Asn Asn Gly Gly
Gly Asn Thr Gly Gly Ser Ala Pro Leu;

25

Val Phe Thr Cys Ser Gly Asn Ser Gly Gly Ser Asn Pro Ser Asn
Pro Asn Pro Pro Thr Pro Thr Phe Ile Thr Gln Val Pro Asn Pro
Thr Pro Val Ser Pro Pro Thr Cys Thr Val Ala Lys;

30 Pro Ala Leu Trp Pro Asn Asn Pro Gln Gln Gly Asn Pro Asn Gln
Gly Gly Asn Asn Gly Gly Asn Gln Gly Gly Asn Gly Gly Cys
Thr Val Pro Lys;

Pro Gly Ser Gln Val Thr Thr Ser Thr Ser Ser Ser Thr Thr
35 Ser Arg Ala Thr Ser Thr Ser Ala Gly Gly Val Thr Ser Ile Thr
Thr Ser Pro Thr Arg Thr Val Thr Ile Pro Gly Gly Ala Ser Thr Thr
Ala Ser Tyr Asn;

47

Glu Ser Gly Gly Gly Asn Thr Asn Pro Thr Asn Pro Thr Asn Pro Thr
Asn Pro Thr Asn Pro Thr Asn Pro Trp Asn Pro Gly Asn Pro Thr Asn
Pro Gly Asn Pro Gly Gly Asn Gly Gly Asn Gly Cys Ser
Pro Leu; or

5

Pro Ala Val Gln Ile Pro Ser Ser Ser Thr Ser Ser Pro Val Asn Gln
Pro Thr Ser Thr Ser Thr Ser Thr Ser Thr Ser Ser Pro Pro
Val Gln Pro Thr Thr Pro Ser Gly Cys Thr Ala Glu Arg

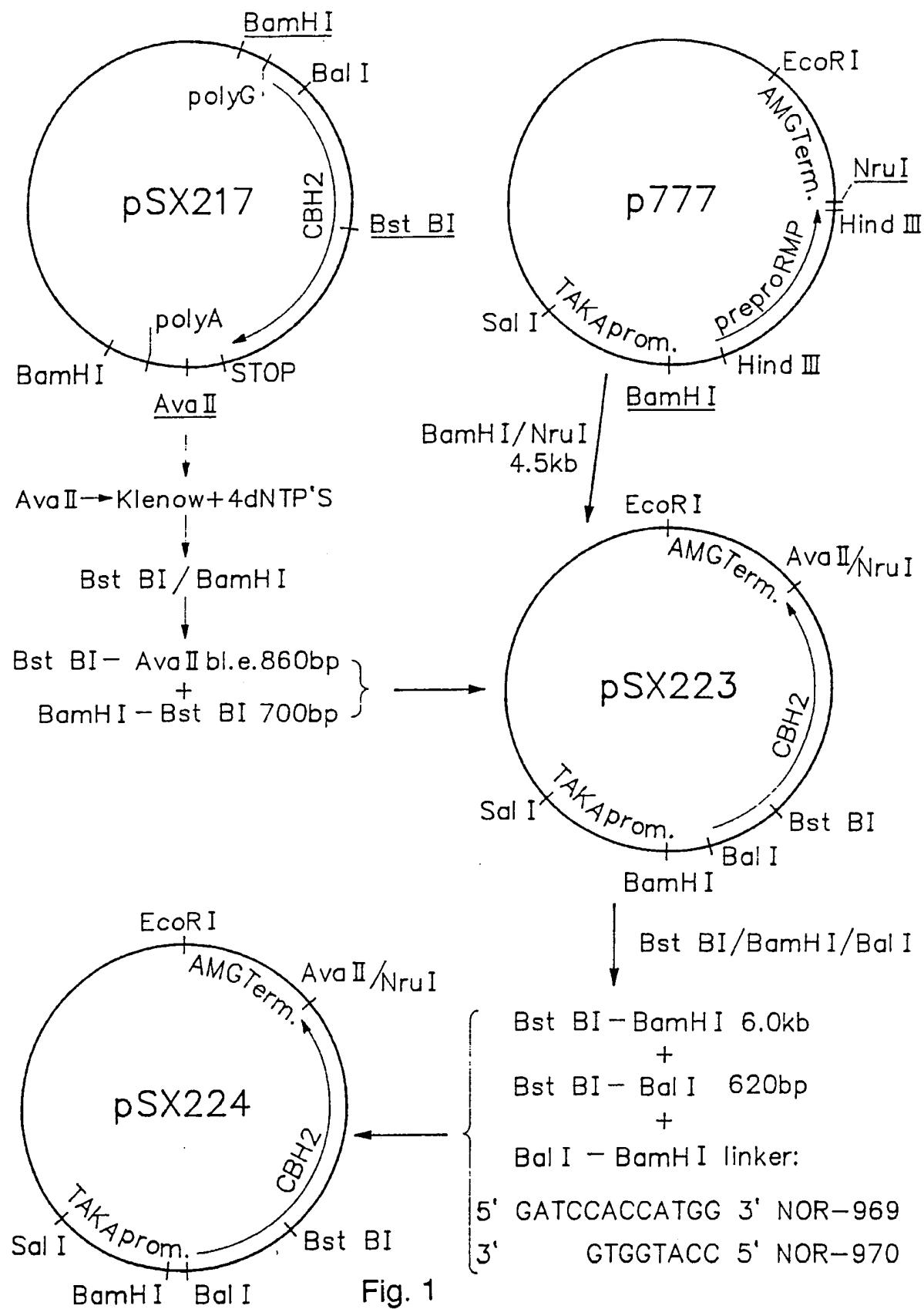


Fig. 1

2/22

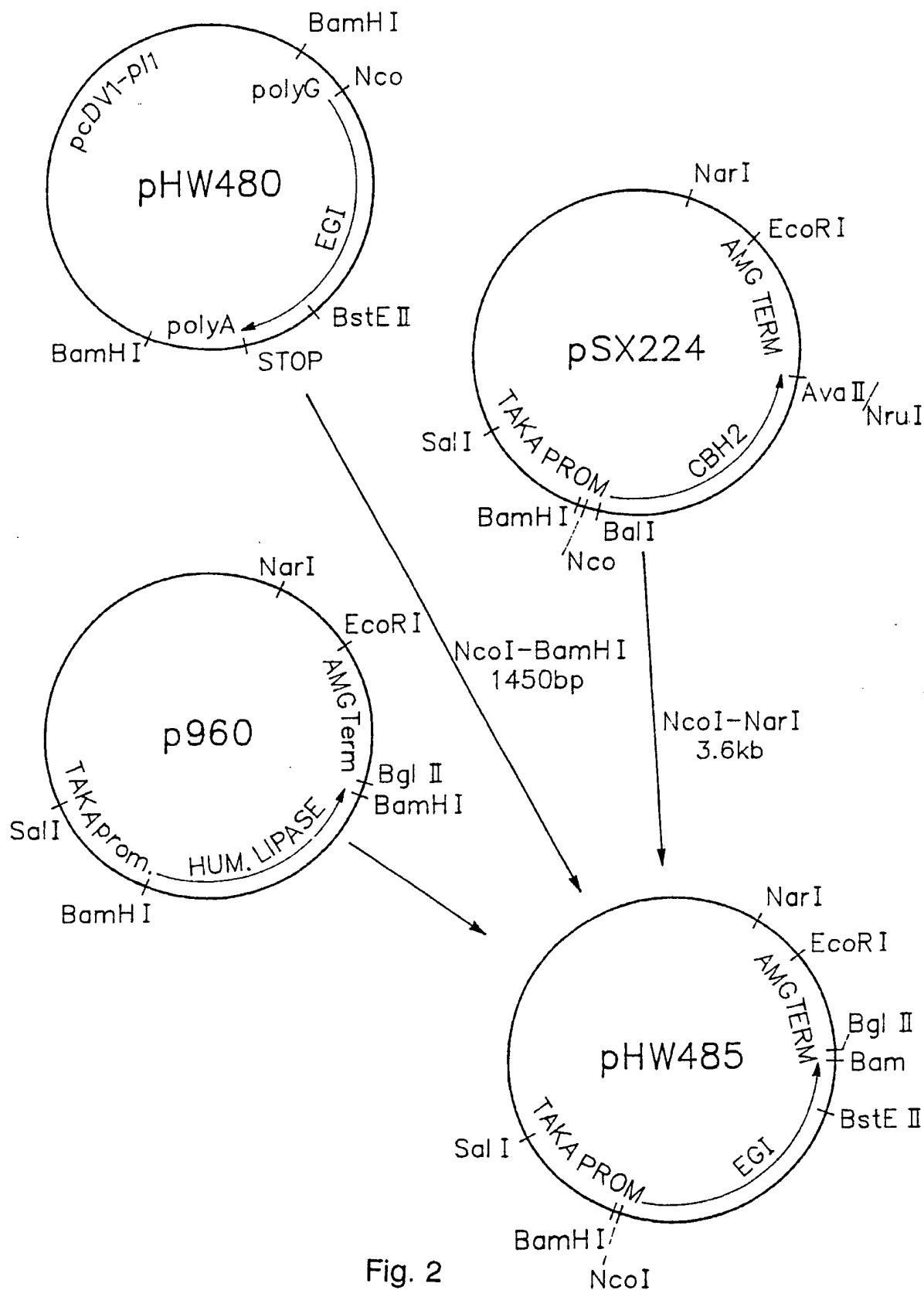


Fig. 2

3/22

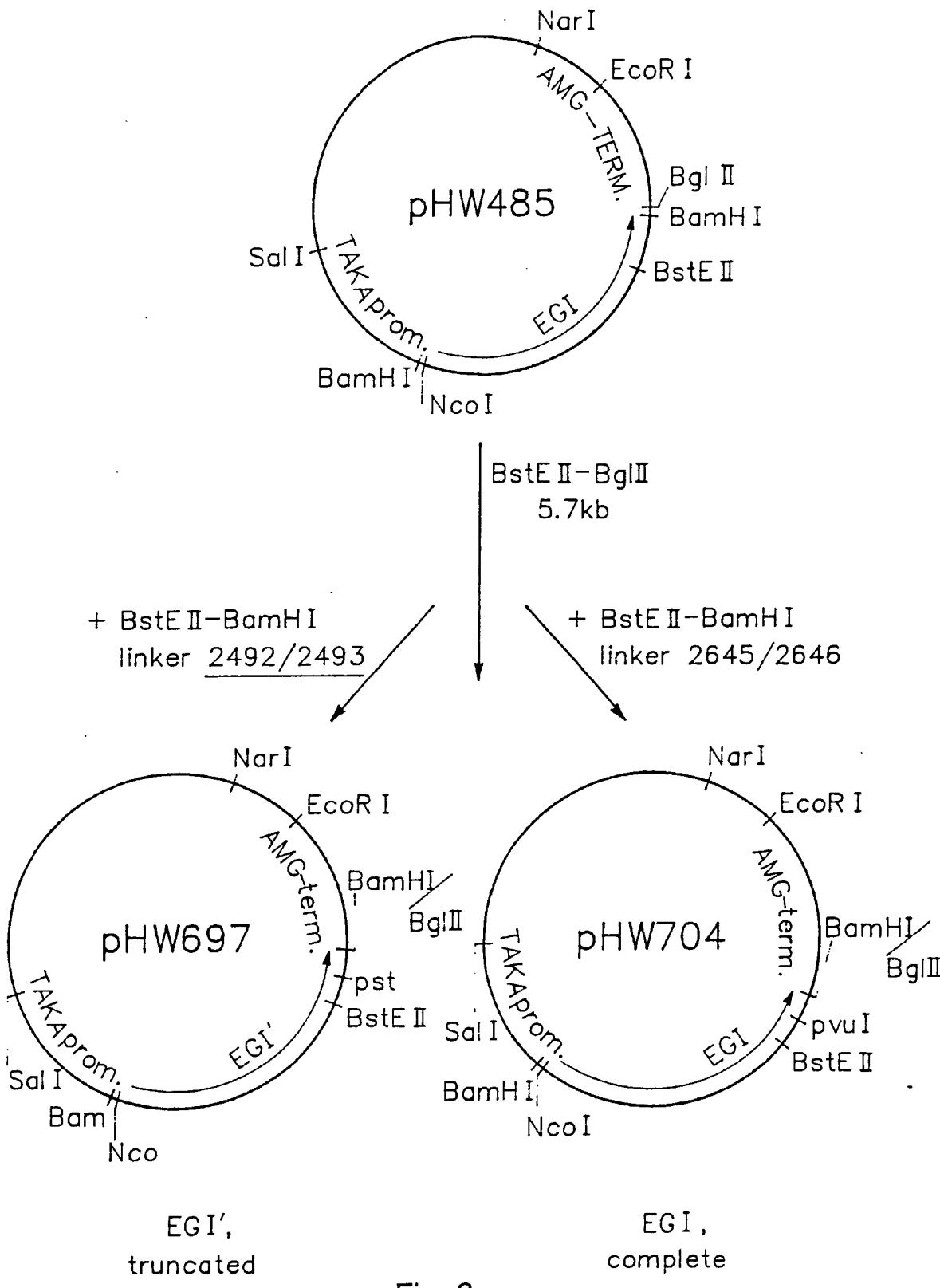


Fig. 3

4/22

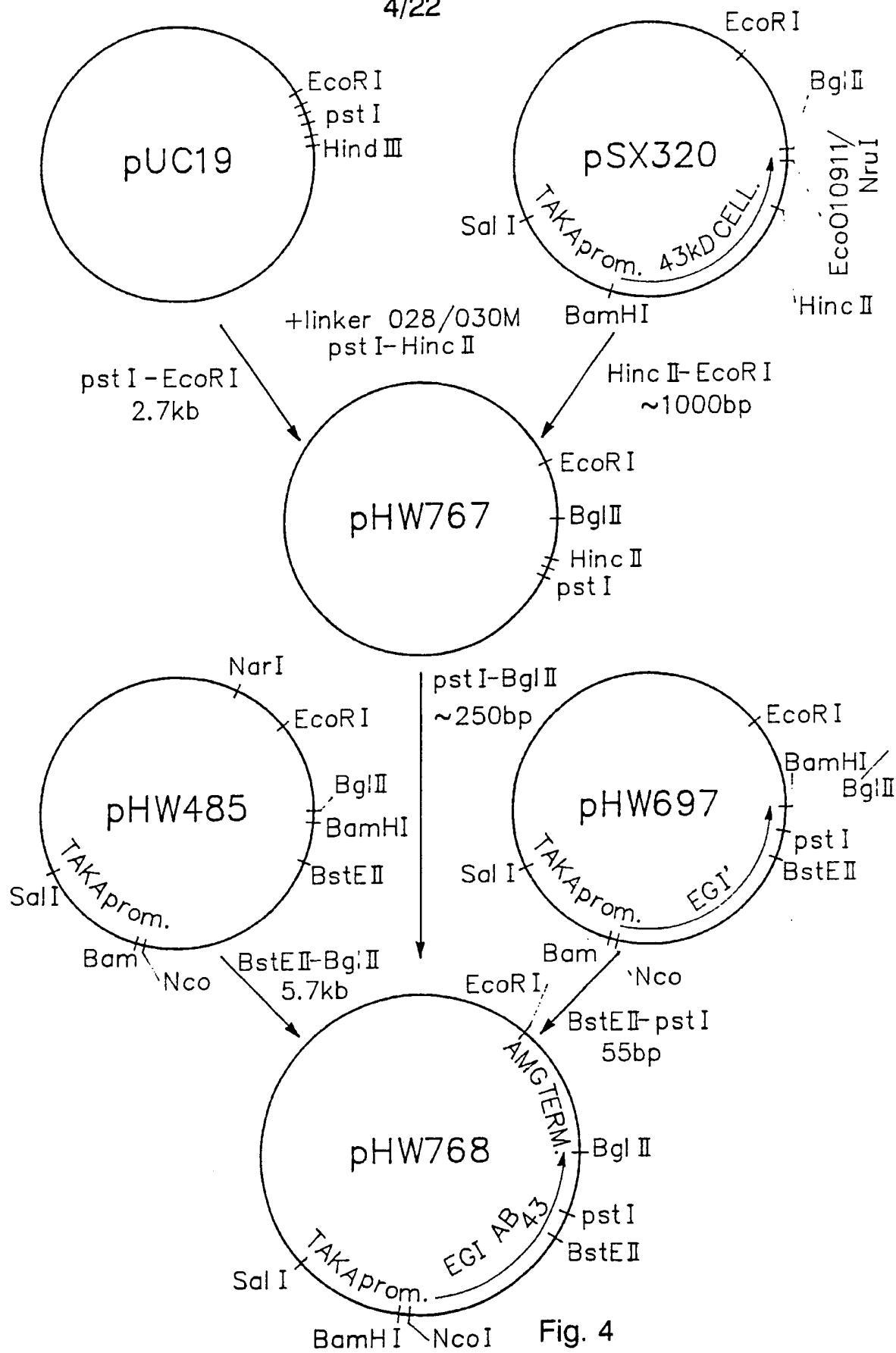


Fig. 4

5/22

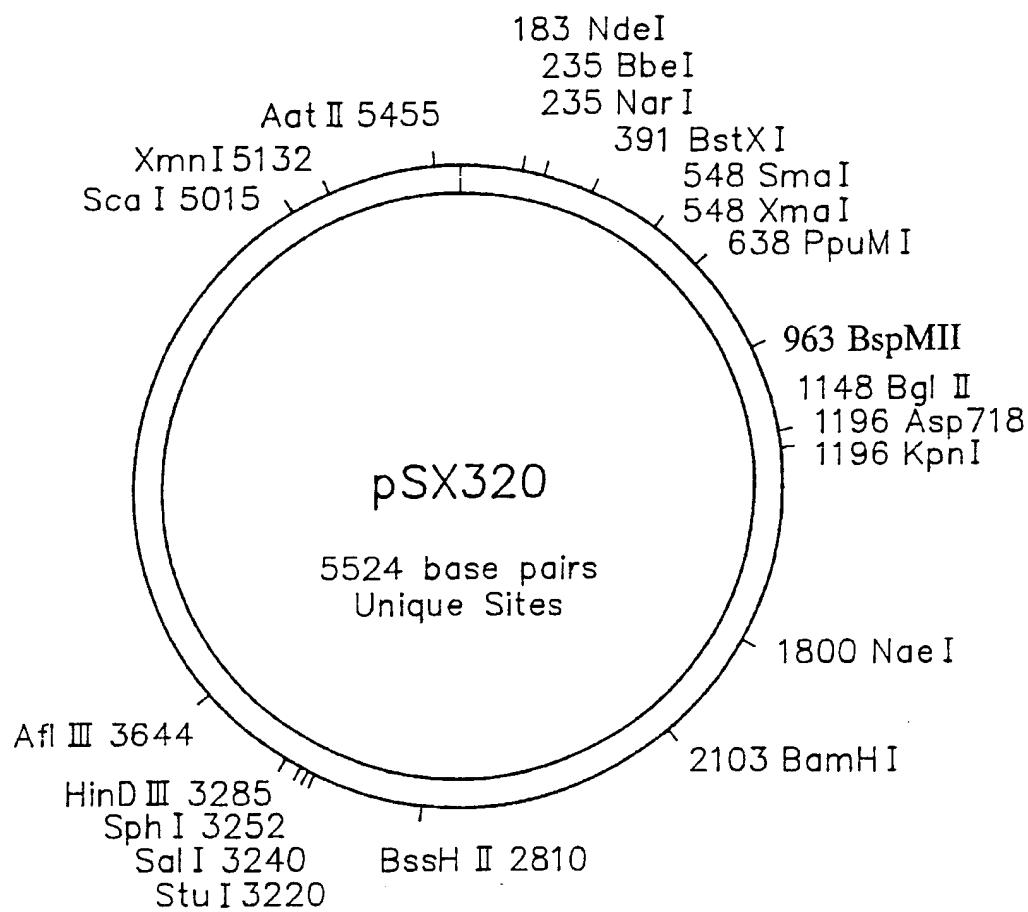
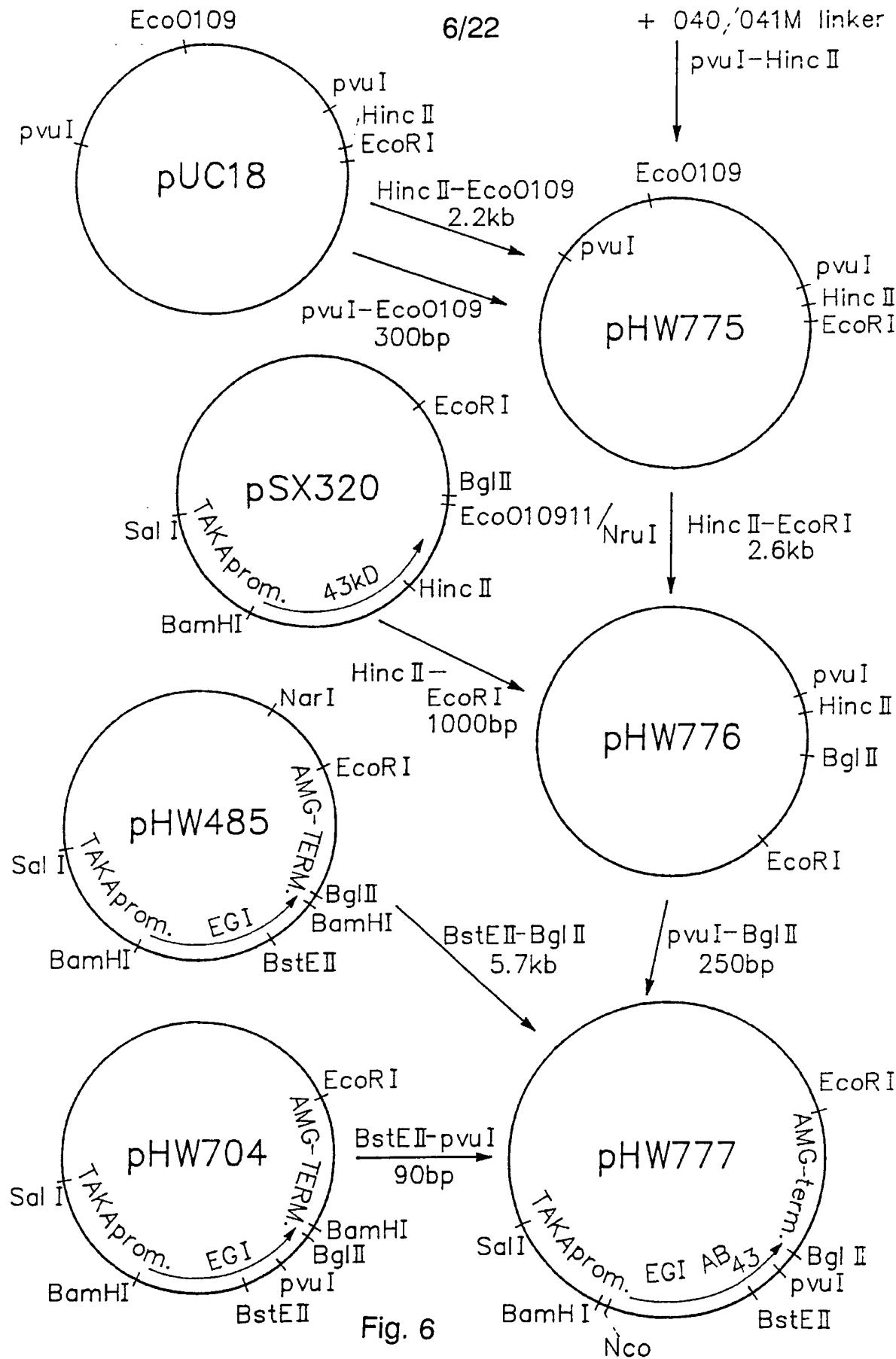


Fig. 5



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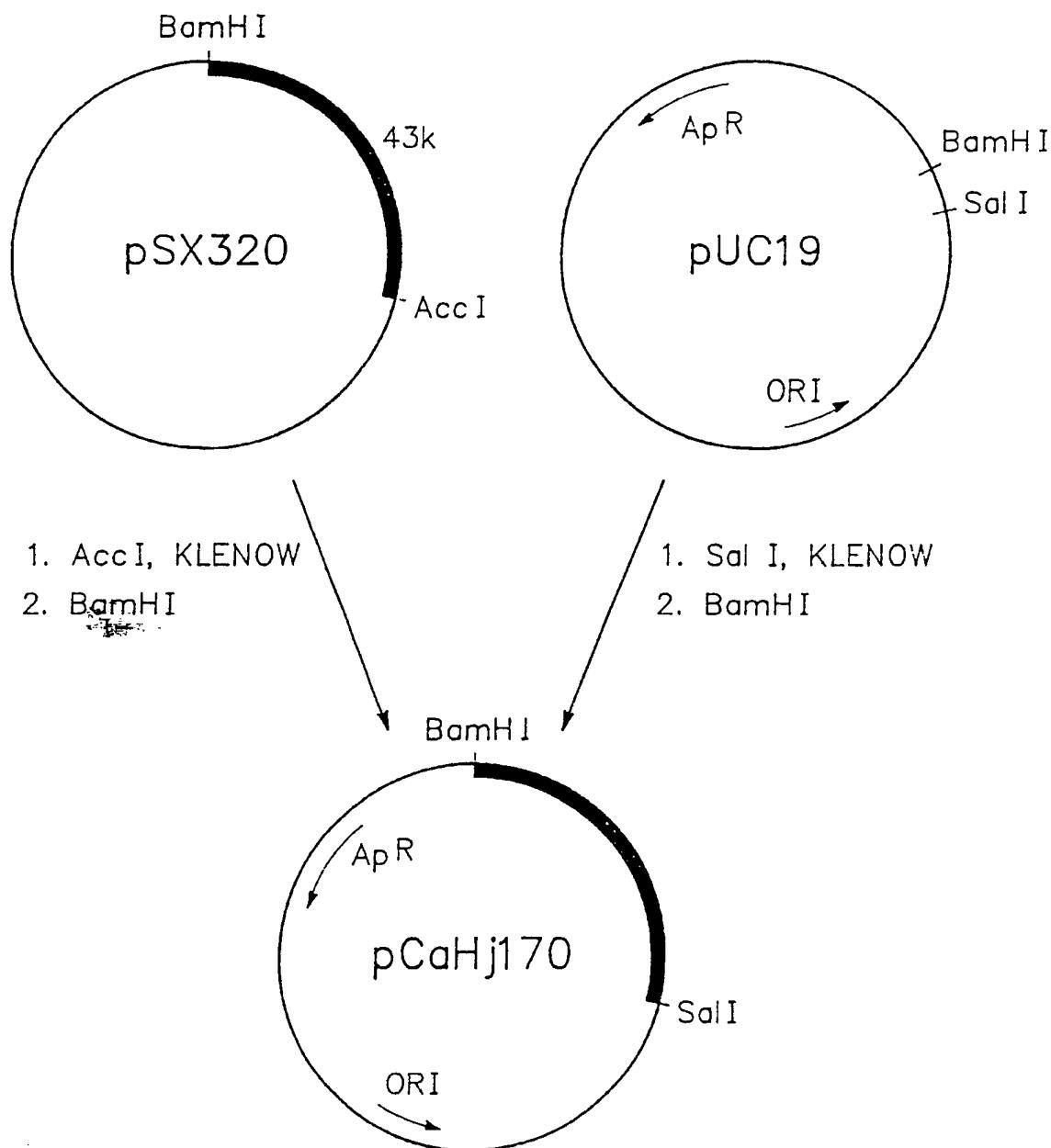
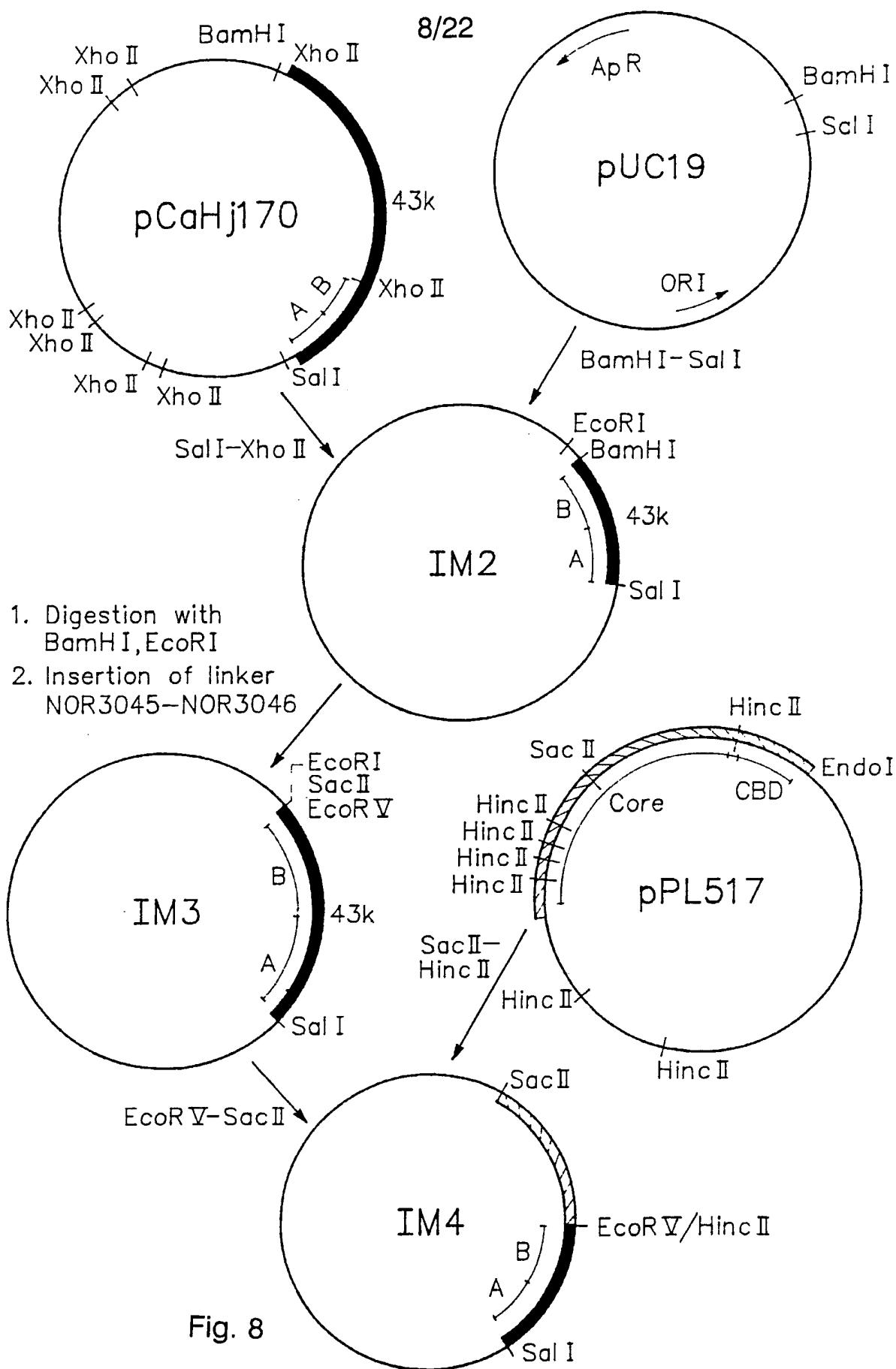
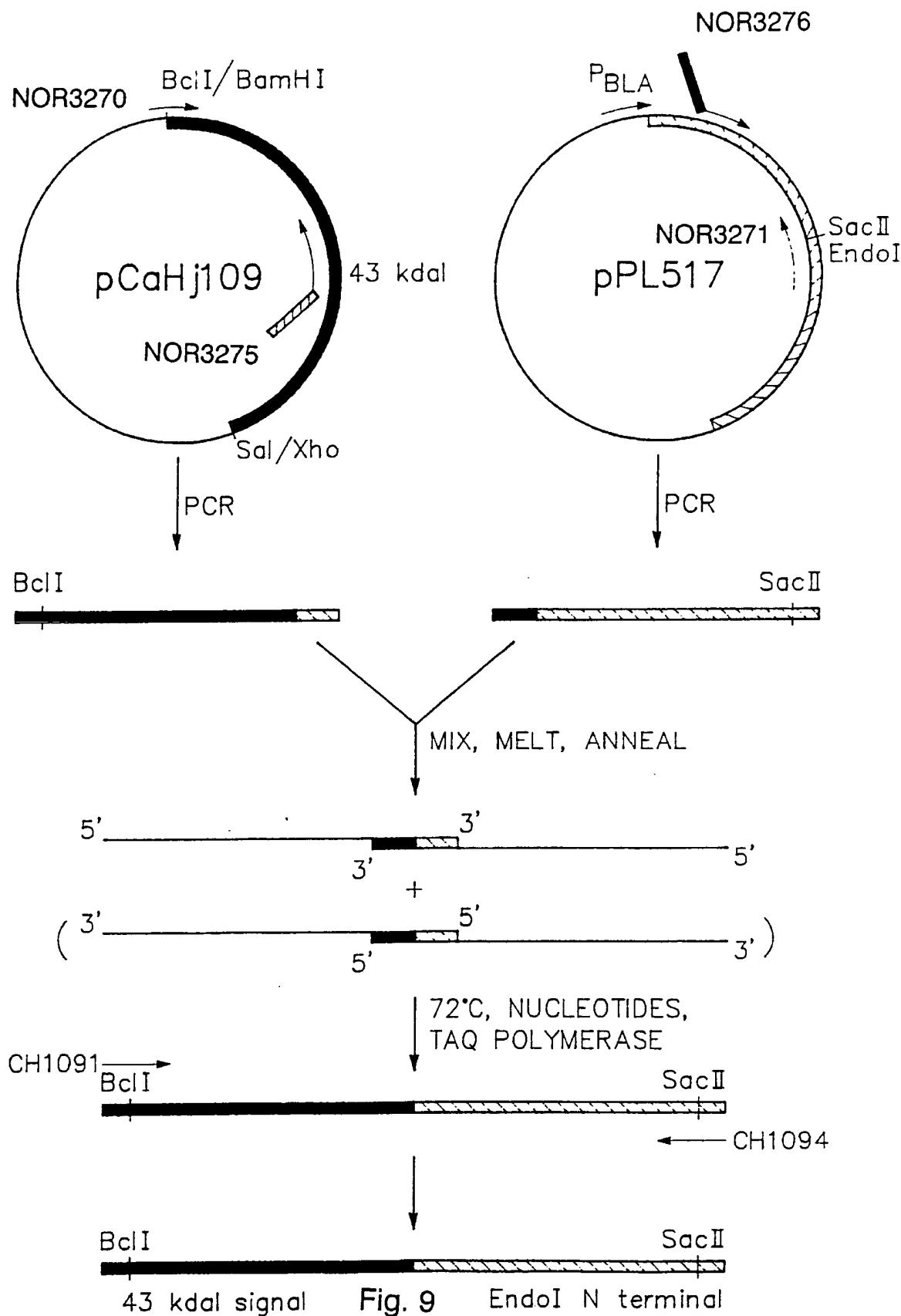


Fig. 7



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10/22

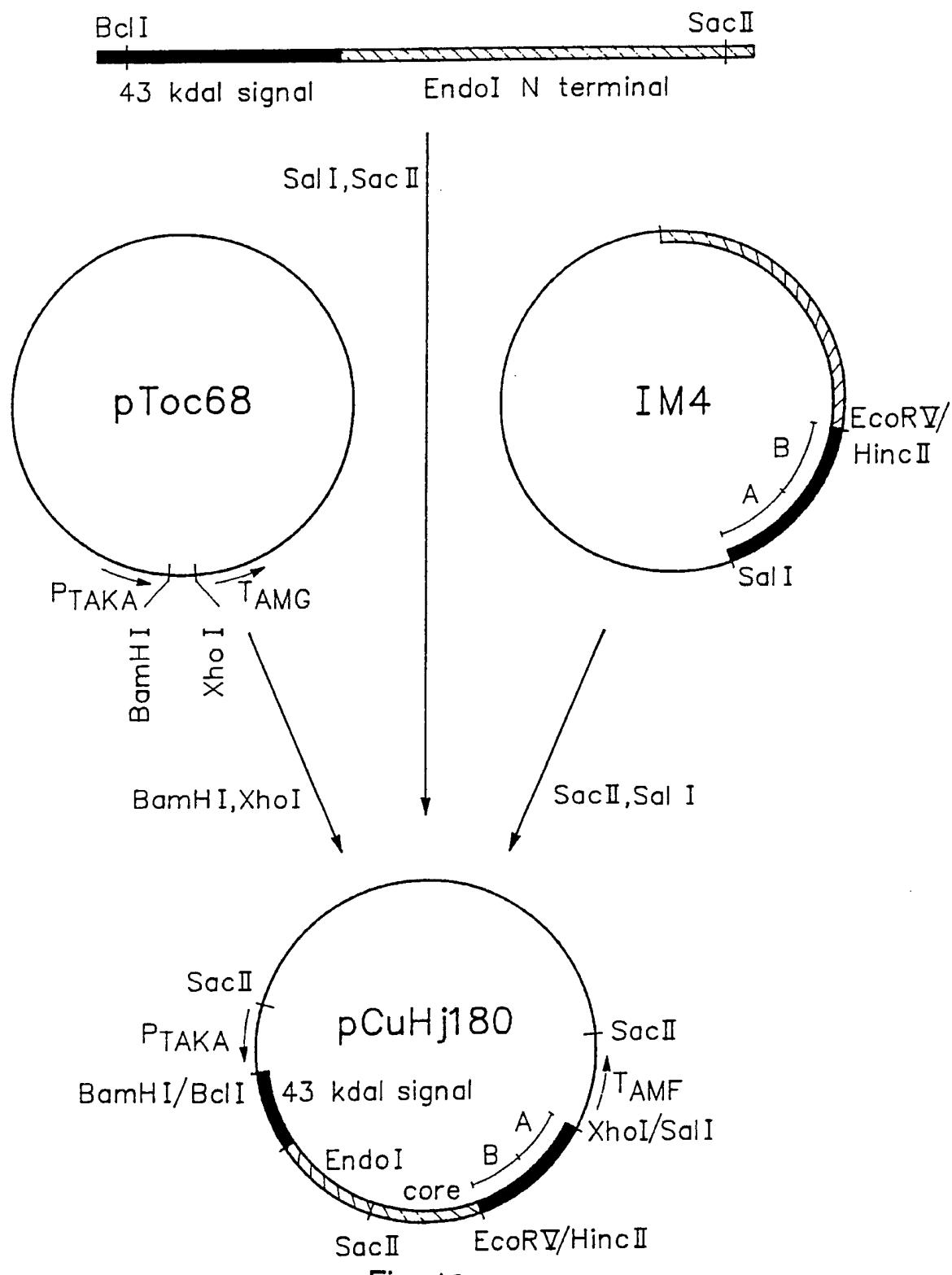


Fig. 10

11/22

agaccggaattcgccggccgcacatctatccaacggtagttcacttcacaatgtatcgc
 M Y R
 atcgtcgcaaccgcctcggttattgccgtcgccgtcaacaggctgtcttgc
 I V A T A S A L I A A A R A Q Q V C S L
 aacaccgagaccaaggctgccttgcactggccaagtgtacatccacgcggctgc
 N T E T K P A L T W S K C T S S G C S D
 gtcaagggtccgttattgtccactggcgtggactcaccagacttctgggtct
 V K G S V V I D A N W R W T H Q T S G S
 accaactgttacaccggaaacaagtggacacccatctgcactgtatggcaagac
 T N C Y T G N K W D T S I C T D G K T C
 GCCGAAAAGTGTCTTGATGGCGCGACTATTCTGGTACCTACGGAAATCACCTCCAGC
 A E K C C L D G A D Y S G T Y G I T S S
 ggcaaccagctcagtctggattcgtcacaacgggtccatcagaacaatcggc
 G N Q L S L G F V T N G P Y S K N I G S
 cgaacacctacccatggagaacgagaacaccatccagatgttccagcttctgg
 R T Y L M E N E N T Y Q M F Q L L G N E
 ttcaccttgcgtatgtctgtatcggtatcggtatcggtatcggtatcggt
 F T F D V D V S G I G C G L N G A P H F
 gtcagcatggacgaggatggcaaggccaagtactccggaaacaaggccgagcca
 V S M D E D G G K A K Y S G N K A G A K
 tacggaaactggcTActGTGATGCCAgTGCCTCGTGTCAAGTTCATCAACGGAGTT
 Y G T G Y C D A Q C P R D V K F I N G V
 GCCAACTCTGAGGGCTGGAAGCCCTCTGACAGTGATGTCAACGCTggtgttaatctg
 A N S E G W K P S D S D V N A G V G N L
 ggcacctgctccccgagatggatatctggaggccaactccatctccaccgc
 G T C C P E M D I W E A N S I S T A F T
 cctcatcctgcaccaagctcacacagcactctgcactggcactctgtgtgg
 P H P C T K L T Q H S C T G D S C G G T
 tactctagtgaccgatatacggtatcgatccgcacgggtgtgatttcaatgc
 Y S S D R Y G G T C D A D G C D F N A Y
 cgtcaggcaacaagacccatctacggctggatccaacttcaacatcgacacc
 R Q G N K T F Y G P G S N F N I D T T K
 aagatgactgttcactcagttccacaaggcAGCAACGGACGTCTTGAGATCACC
 K M T V V T Q F H K G S N G R L S E I T
 CGTCTGTACGTCCAGAACGGCAAGGTCTTGCCAACTCAGAGTCCAAGATTGCAGG
 R L Y V Q N G K V I A N S E S K I A G N
 CCCGGTAGCTCTCACCTCTGACTCTGCTCCAAAGCAGAAGAGCGTCTTGCGATATC
 P G S S L T S D F C S K Q K S V F G D I
 GATGACTTCTCTAAAGAAGGGTGGCTGGAACGgCATGAGCGATGCTCTCTGCC
 D D F S K K G G W N G M S D A L S A P M
 GTTCTTGTATGTCCTCTGGCACGACCACCTCAACATGCtCTGGCTgGACtacc
 V L V M S L W H D H H S N M L W L D S T
 taccggacttaccaagggtggatctcaacgaggcttgcgttaccac
 Y P T D S T K V G S Q R G S C A T T S G
 aagccctccgacccatggcgagatgttccaaactccaagggttccatcca
 K P S D L E R D V P N S K V S F S N I K
 TTGGTCCCCATGGAAAGCACCTACAAGAGCGACGGCACCACCCCCAACCCCC
 F G P I G S T Y K S D G T T P N P P A S
 AGCAGCACCACGGTTCTTCACTCCCACCAACCCCCCTGCCGGTAGCGTC
 S S T T G S S T P T N P P A G S V D Q W
 GGACAGTGCGGTGGCCAgactacagcggcccccacgac
 G Q C G G Q N Y S G P T T C K S P F T C
 aagaagatcaacgacttctactccaggatgtcagtaaagg
 K K I N D F Y S Q C Q .
 gagattgagaaacgatgtgatgtggacatcaaggagaagtgtgtggat
 gagat

ttgatgtggaggac

Fig. 11

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gaattcgcggccgttcgaagcatcagctattgagatcagtcaaaatgcatacc
M H T
cttcgggttctcctcgcttcgtccccgtgtccgccttgctcaggctccatctgggga
L S V L L A L A P V S A L A Q A P I W G
cagtgcgggtggcaatgggtggaccgggtgctacaacctgcgtactgggtctgaagtgtgag
Q C G G N G W T G A T T C A S G L K C E
aagatcaacgactggtaactatcagtgtttccctggatctggaggatctgaaccccagcct
K I N D W Y Y Q C V P G S G G S E P Q P
tcgtcaactcagggtggcactcctcagcctactggcgtaacagcggcggcactgg
S S T Q G G G T P Q P T G G N S G G T G
ctcgacgccaattcaaggccaaggcaagcagtaacttggtaccgagattgaccactac
L D A K F K A K G K Q Y F G T E I D H Y
cacctaacaacaatcctctgtatcaacattgtcaaggcccagttggccaagtgacatgc
H L N N N P L I N I V K A Q F G Q V T C
gagaacagcatgaagtggatgccattgagccttcacgcaactccttacacccatcagaac
E N S M K W D A I E P S R N S F T F S N
gctgacaaggctcgacttcgcccactcagaacggcaagctcatccgtgGCCACACTCTT
A D K V V D F A T Q N G K L I R G H T L
CTCTGGCACTCTCAGCTGCCAGTGGTTAGAACATCAACGATCGCTCACCCCTCAC
L W H S Q L P Q W V Q N I N D R S T L T
GCGGTCACTCGAGAACACCAGTCAGAACGACCATGGTCACCCGCTACAAGGGCAAGATCCTCCAG
A V I E N H V K T M V T R Y K G K I L Q
TGGGACGTTGTCACAAACGAGATCTCGCTGAGGACGTAACCTCCGCGACAGTGTCTTC
W D V V N N E I F A E D G N L R D S V F
AGCCGAGTTCTCGGTGAGGACTTGTGCGTTGCTTCCGCGCTGCCCGCGCCGCTGAT
S R V L G E D F V G I A F R A A R A A D
CCCGCTGCCAACGCTCTACATCAACGATTATAACCTCGACAAGTCCGACTATGCTAACGGTC
P A A K L Y I N D Y N L D K S D Y A K V
ACCCGCGGAATGGTCGCTCACGTTAAAGTGGATTGCTGCTGGTATTCCCATCGACGGT
T R G M V A H V N K W I A A G I P I D G
ATTGGATCTCAGGGCCATCTGCTGCTCCTAGTGGCTGGAACCCCTGCCTCTGGTTCCT
I G S Q G H L A A P S G W N P A S G V P
GCTGCTCTCCGAGCTCTGCGCCCTCGGACGCCAAGGAGATTGCTATcactgagcttgat
A A L R A L A A S D A K E I A I T E L D
attgccccgtgcccagtgcataacgattaccttaactgtcatgaacgcgttgcctggccgttccc
I A G A S A N D Y L T V M N A C L A V P
aagtgtgtcgccatcactgtctgggtgtctgtacaaggactcgtggcggatctgg
K C V G I T V W G V S D K D S W R P G D
aaccccctccctacgacacgcaactaccagccaaaggctgtttcaatgccttggctaac
N P L L Y D S N Y Q P K A A F N A L A N
gctctgtgagctgttgttatgtcgctggatcatacaacgaaacgtccttagttgga
A L .
taaagcgtttaggtatgtatgtatgtcgctggatcatacaacgaaacgtccttagttgga

Fig. 12

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gaattcgcggccgcctagataagtactacacctgtatcttgcataatcttcatcatgaag
M K
tctctctcaactcatcctctcagccctggctgtccaggcgctgttgctcaaaccggcgcac
S L S L I L S A L A V Q V A V A Q T P D
aaggccaaggagcagcaccccaagctcgagacccatccgctgcaccaaggccctctggctgc
K A K E Q H P K L E T Y R C T K A S G C
aagaagcaaaccactacatcgccgaCgcaggattcacggcattCgcagaagcgCC
K K Q T N Y I V A D A G I H G I R R S A
GGCTCGGGTGA C T G G G T C A A A G C C C A C G C C A C G C T G C C C G A T G A G G C A T C C T G C
G C G D W G Q K P N A T A C P D E A S C
GCTAAGAACTGTATCCTCAGTGGTATGGACTCAAACGCTTACAAGAACGCTGGTATCACT
A K N C I L S G M D S N A Y K N A G I T
ACTTCTGGCAACAAGCTCGTCTTCAGCAGCTTATCAACAACCAGCTTGTTCCTCCTCGG
T S G N K L R L Q Q L I N N Q L V S P R
GTTTATCTGCTTGAGGAGAACAGAACAGTATGAGATGCTTCAGCTCACTGGTACTGAA
V Y L L E E N K K K Y E M L H L T G T E
TTCTCTTCGACGTTGAGATGGAGAACAGCTTCCTTGTGGTATGAATGGTGCCTTGTACCTT
F S F D V E M E K L P C G M N G A L Y L
TCCGAGATGCCACAGGATGGTAAGAGCACAGGCCAACAGCAAGGCTGGTGCCTAC
S E M P Q D G G K S T S R N S K A G A Y
TATGGTGCCTGGATACTGTGATGCTCAGTGCCTACGT^CactcctttcATCAACGGAGTTGGC
Y G A G Y C D A Q C Y V T P F I N G V G
AACATCAAGGGACAGGGTGTCTGCTGAACGAGCTCGACATCTGGGAGGCCAACTCCCGC
N I K G Q G V C C N E L D I W E A N S R
GCAACTCACATTGCTCCTCACCTTGAGCAAGCCCCGCTCTACGGCTGCACAGGGCAGT
A T H I A P H P C S K P G L Y G C T G D
GAGTGGCGCAGCTCCGGTTCTGCGACAAGGCCGGCTGGCTGGAACACAAACCGCATC
E C G S S G I C D K A G C G W N H N R I
AACGTGACCGACTTCTACGGcccgqCAAGCAGTACAAGGTCGACAGCACCCGCAAGTTC
N V T D F Y G R G K Q Y K V D S T R K F
ACCGTGACATCTCAGTTGCGCCAACAAGCAGGGTGATCTCATCGAGCTGCACCGCCAC
T V T S Q F V A N K Q G D L I E L H R H
TACATCCAGGACAACAAGGTCAtcgagtctgtcgtaacatctccggccctcccaag
Y I Q D N K V I E S A V V N I S G P P K
atcaacttcatcaatgacaagtaactgcgcgtccaccggcgccaaacgagttacatgcgcctc
I N F I N D K Y C A A T G A N E Y M R L
ggcggtactaagcaaattggcgatgccatgtcccggaaatgggtctcgccatgagcgtc
G G T K Q M D A M S R G M V L A M S V
tgggtggagcgaggggtgattcatggcctggatcagggtgttgctggaccctgtgac
W W S E G D F M A W L D Q G V A G P C D
gccaccggaggcgatcccaagaacatcgtaaggtgcagccccaaaccctgaagtgacattt
A T E G D P K N I V K V Q P N P E V T F
agcaacatcagaattggagagattggatctacttcatcggtcaaggctcctcggtatcct
S N I R I G E I G S T S S V K A P A Y P
ggtcctcaccgctgtaaaaacatcaaacaacaccgtgtccaatatggATCTTAGTGTCC
G P H R L .
ACTTGCTGGGAAGCTATTGGAGCACATATGCAAAACAGATGTCCACTAGCTGACACGTA
TGTGGGGCAAAAAATCTTTCTAGGATAGGAGAACATATTGGGTGTTGGACTTGTA
TATAAAATGATACATTTTCAATTATATTATTTCAACATATTTATTCACGAAAAAAA
AAAAAAAAAAAAAAAAAAAAAA

Fig. 13

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10	20	30	40	50	60
TTTCTTCGTCGAGCTCGAGTCGTCCGCCGTCTCCTCCTCCTCCTCCAGTCTTGAG					
70	80	90	100	110	120
TTCCTTCGACCTGCAGCGTCCTGAACAACACTCGCTCTAGCTCAACAACCATGGCTCGCGGT					
MetAlaArgGly					
130	140	150	160	170	180
ACCGCTCTCCTCGGCCTGACCGCGCTCCTCCTGGGGCTGGTCAACGGCCAGAACCTGGT					
ThrAlaLeuLeuGlyLeuThrAlaLeuLeuLeuGlyLeuValAsnGlyGlnLysProGly					
190	200	210	220	230	240
GAGACCAAGGAGGTTCACCCCCAGCTCACGACCTCCGCTGCACGAAGAGGGGTGGTTGC					
GluThrLysGluValHisProGlnLeuThrThrPheArgCysThrLysArgGlyGlyCys					
250	260	270	280	290	300
AAGCCGGCGACCAACTTCATCGTGCTTGACTCGCTGTCGCACCCCATCCACCGCGCTGAG					
LysProAlaThrAsnPheIleValLeuAspSerLeuSerHisProIleHisArgAlaGlu					

Fig. 14A

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310 320 330 340 350 360

| | | | | |

GGCCTGGCCCTGGCGGCTGCAGCGACTGGGGCAACCCGCCGCCAAGGACGTCTGCCCG

GlyLeuGlyProGlyGlyCysGlyAspTrpGlyAsnProProProLysAspValCysPro

370 380 390 400 410 420

| | | | | |

GACGTCGAGTCGTGCCAAGAACTGCATCATGGAGGGCATCCCCACTACAGCCAGTAC

AspValGluSerCysAlaLysAsnCysIleMetGluGlyIleProAspTyrSerGlnTyr

430 440 450 460 470 480

| | | | | |

GGCGTCACCACCAACGGCACCAAGCCTCCGCCGCAGCACATCCTCCCCGACGGCCCGTAC

GlyValThrThrAsnGlyThrSerLeuArgLeuGlnHisIleLeuProAspGlyArgVal

490 500 510 520 530 540

| | | | | |

CCGTCGCCGCGTGTCTACCTGCTCGACAAGACGAAGCGCCGCTATGAGATGCTCCACCTG

ProSerProArgValTyrLeuLeuAspLysThrLysArgArgTyrGluMetLeuHisLeu

550 560 570 580 590 600

| | | | | |

ACCGGCTTCGAGTTCACCTCGACGTCGACGCCACCAAGCTGCCCTGCGGCATGAACAGC

ThrGlyPheGluPheThrPheAspValAspAlaThrLysLeuProCysGlyMetAsnSer

610 620 630 640 650 660

| | | | | |

GCTCTGTACCTGTCCGAGATGCACCCGACCGGTGCCAAGAGCAAGTACAACCTCCGGCGGT

AlaLeuTyrLeuSerGluMetHisProThrGlyAlaLysSerLysTyrAsnSerGlyGly

Fig. 14B

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670 680 690 700 710 720

| | | | | |

GCCTACTACGGTACTGGCTACTGCGATGCTCAGTGCTTCGTGACGCCCTCATCAACGGC

AlaTyrTyrGlyThrGlyTyrCysAspAlaGlnCysPheValThrProPheIleAsnGly

730 740 750 760 770 780

| | | | | |

TTGGGCAACATCGAGGGCAAGGGCTCGTGTGCAACGAGATGGATATCTGGGAGGTCAAC

LeuGlyAsnIleGluGlyLysGlySerCysCysAsnGluMetAspIleTrpGluValAsn

790 800 810 820 830 840

| | | | | |

TCGCGCGCCTCGCACGTGGTTCCCCACACCTGCAACAAGAAGGGCCTGTACCTTGCAG

SerArgAlaSerHisValValProHisThrCysAsnLysLysGlyLeuTyrLeuCysGlu

850 860 870 880 890 900

| | | | | |

GGTGAGGAGTGCACCGCTTCGAGGGTGTGCGACAAGAACGGCTGCGCTGGAAACAAC

TACGlyGluGluCysAlaPheGluGlyValCysAspLysAsnGlyCysGlyTrpAsnAsnTyr

910 920 930 940 950 960

| | | | | |

CGCGTCAACGTGACTGACTACTACGGCCGGGGCGAGGAGTTCAAGGTCAACACCCTCAAG

ArgValAsnValThrAspTyrTyrGlyArgGlyGluGluPheLysValAsnThrLeuLys

970 980 990 1000 1010 1020

| | | | | |

CCCTTCACCGTCGTCACTCAGTTCTGGCCAACCGCAGGGCAAGCTCGAGAAGATCCAC

ProPheThrValValThrGlnPheLeuAlaAsnArgArgGlyLysLeuGluLysIleHis

Fig. 14C

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1030 1040 1050 1060 1070 1080

| | | | | |

CGCTTCTACGTGCAGGACGGCAAGGTATCGAGTCCTCTACACCAACAAGGAGGGAGTC
ArgPheTyrValGlnAspGlyLysValIleGluSerPheTyrThrAsnLysGluGlyVal

1090 1100 1110 1120 1130 1140

| | | | | |

CCTTACACCAACATGATCGATGACGAGTTCTGCGAGGCCACCGGCTCCGCAAGTACATG
ProTyrThrAsnMetIleAspAspGluPheCysGluAlaThrGlySerArgLysTyrMet

1150 1160 1170 1180 1190 1200

| | | | | |

GAGCTCGGCCACCCAGGGCATGGCGAGGCCCTCACCCGGCATGGTCTGGCCATG
GluLeuGlyAlaThrGlnGlyMetGlyGluAlaLeuThrArgGlyMetValLeuAlaMet

1210 1220 1230 1240 1250 1260

| | | | | |

AGCATCTGGTGGGACCAGGGCGAACATGGAGTGGCTCGACCACGGCGAGGCCGGCCCC
SerIleTrpTrpAspGlnGlyGlyAsnMetGluTrpLeuAspHisGlyGluAlaGlyPro

1270 1280 1290 1300 1310 1320

| | | | | |

TGCGCCAAGGGCGAGGGCGCCCCGTCCAACATTGTCAGGTTGAGCCCTCCCCGAGGTC
CysAlaLysGlyGluGlyAlaProSerAsnIleValGlnValGluProPheProGluVal

1330 1340 1350 1360 1370 1380

| | | | | |

ACCTACACCAACCTCCGCTGGGGCGAGATCGGCTCGACCTACCAGGAGGTTCAAGAAGCCT
ThrTyrThrAsnLeuArgTrpGlyGluIleGlySerThrTyrGlnGluValGlnLysPro

Fig. 14D

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1390 1400 1410 1420 1430 1440

| | | | | |

AAGCCCAAGCCCCGCCACGGCCCCCGGAGCGACTAAGTGGTGATGGGATAGAGGGATAGA

LysProLysProGlyHisGlyProArgSerAspEND

1450 1460 1470 1480 1490 1500

| | | | | |

ATAGTGGATAGCACATAGATCGGCGGTTTGGATAGTTAACATACATTCCGTTGCCGTTGT

1510

GAAAAAAA - poly-A

Fig. 14E

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10 20 30 40 50 60

ATGC GTT CCT CCCCC CCT CC CGT CC GCG CC GT GG CC GC CT GC CG GT TT GG CC CT

MET Arg Ser Ser Pro Leu Leu Pro Ser Ala Val Val Ala Ala Leu Pro Val Leu Ala Leu
43 kdal signal peptide and N terminal

70 80 90 100 110 120

GCC GCT GAT GGC AGG AGT GAT GTC ACT TT CAC GAT TA AT A CG CAG T CG G AAC GT G C AG CG

Ala Ala Asp Gly Arg Ser Asp Val Thr Phe Thr Ile Asn Thr Gln Ser Glu Arg Ala Ala
N terminal

130 140 150 160 170 180

ATC AGC CCC AAT ATT TAC GG A ACCA AT CAG GAT CT GAG CGG AC GG AGA ACT GGT CAT CC

Ile Ser Pro Asn Ile Tyr Gly Thr Asn Gln Asp Leu Ser Gly Thr Glu Asn Trp Ser Ser

190 200 210 220 230 240

CGC AGG CT CGG AGG CA ACC GG CT GAC GGG TT ACA ACT GGG AGA ACA AC G CAT CC AG CG CC

Arg Arg Leu Gly Gly Asn Arg Leu Thr Gly Tyr Asn Trp Glu Asn Asn Ala Ser Ser Ala

250 260 270 280 290 300

GGA AGGG ACT GG CCT TC ATT AC AG CG AT GATT T CT GCG G CA AC GG TG GT TT CC AG AC

Gly Arg Asp Trp Leu His Tyr Ser Asp Asp Phe Leu Cys Gly Asn Gly Gly Val Pro Asp
Endo 1 core

310 320 330 340 350 360

ACCG ACT GCG ACA AG CCG GGG CG GT TG TT ACC GCT TT CAC GATA AA AT CTT GG AGA AT

Thr Asp Cys Asp Lys Pro Gly Ala Val Val Thr Ala Phe His Asp Lys Ser Leu Glu Asn

370 380 390 400 410 420

GG AGC TT ACT CC ATT GT AAC GCT GCA AA AT GG CG GG TT AT GT GT CC CG G ATA AGA AC GG

Gly Ala Tyr Ser Ile Val Thr Leu Gln MET Ala Gly Tyr Val Ser Arg Asp Lys Asn Gly

430 440 450 460 470 480

CC AGT TG AC GAG AGT GAG AC GG CT CC GT ACC GCG TT GG G ATA AGG T CG AG T TT G C AAA

Pro Val Asp Glu Ser Glu Thr Ala Pro Ser Pro Arg Trp Asp Lys Val Glu Phe Ala Lys

Fig. 15A

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490 500 510 520 530 540
 AATGCGCCGTTCTCCCTTCAGCCTGATCTGAACGACGGACAAGTGTATATGGATGAAGAA
 AsnAlaProPheSerLeuGlnProAspLeuAsnAspGlyGlnValTyrMETAspGluGlu

550 560 570 580 590 600
 GTTAACCTCCTGGTCAACCGGTATGGAAACGCTTCAACGTCAACGGGCATCAAAGCGTAT
 ValAsnPheLeuValAsnArgTyrGlyAsnAlaSerThrSerThrGlyIleLysAlaTyr

610 620 630 640 650 660
 TCGCTGGATAACGAGGCCGGCGCTGTGGTCTGAGACGCATCCAAGGATTCCGGAGCAG
 SerLeuAspAsnGluProAlaLeuTrpSerGluThrHisProArgIleHisProGluGln

670 680 690 700 710 720
 TTACAAGCGGCAGAACTCGTCGCTAACGAGCATCGACTTGTCAAAGGCGGTGAAGAACGTC
 LeuGlnAlaAlaGluLeuValAlaLysSerIleAspLeuSerLysAlaValLysAsnVal

730 740 750 760 770 780
 GATCCGCATGCCGAAATATCGGTCCCTGCCCTTACGGTTTCGGCGCATATTGTCTCTG
 AspProHisAlaGluIlePheGlyProAlaLeuTyrGlyPheGlyAlaTyrLeuSerLeu

790 800 810 820 830 840
 CAGGACGCACCGGATTGCCGAGTTGCAAGGCAACTACAGCTGGTTATCGATTACTAT
 GlnAspAlaProAspTrpProSerLeuGlnGlyAsnTyrSerTrpPheIleAspTyrTyr

850 860 870 880 890 900
 CTGGATCAGATGAAGAATGCTCATACGCAGAACGGCAAAAGATTGCTCGATGTGCTGGAC
 LeuAspGlnMETLysAsnAlaHisThrGlnAsnGlyLysArgLeuLeuAspValLeuAsp

910 920 930 940 950 960
 GTCCACTGGTATCCGGAAGCACAGGGCGGAGGCCAGCGAATCGTCTTGCGGGGGCGGCG
 ValHisTrpTyrProGluAlaGlnGlyGlyGlnArgIleValPheGlyGlyAlaGly

Fig. 15B

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970 980 990 1000 1010 1020

AATATCGATAACGCAAGGCTCGCTACAAGCGCCAAGATCGCTATGGGATCCGGCTTAC
AsnIleAspThrGlnLysAlaArgValGlnAlaProArgSerLeuTrpAspProAlaTyr

1030 1040 1050 1060 1070 1080

CAGGAAGACAGCTGGATCGGCACATGGTTTCAGCTACTTGCCCTTAATTCCGAAGCTG
GlnGluAspSerTrpIleGlyThrTrpPheSerSerTyrLeuProLeuIleProLysLeu

1090 1100 1110 1120 1130 1140

CAATCTTCGATTCAAGACGTATTATCCGGTACGAAGCTGGCGATCACAGAGTTCAGCTAC
GlnSerSerIleGlnThrTyrTyrProGlyThrLysLeuAlaIleThrGluPheSerTyr

1150 1160 1170 1180 1190 1200

GGCGGAGACAATCACATTCGGGAGGCATAGCTACCGCGGACCGCGCTCGGCATTTTGGA
GlyGlyAspAsnHisIleSerGlyGlyIleAlaThrAlaAspAlaLeuGlyIlePheGly

1210 1220 1230 1240 1250 1260

AAATATGGCGTTATGCCCGAATTACTGGCAGACGGAGGACAATACCGATTATACCAGC
LysTyrGlyValTyrAlaAlaAsnTyrTrpGlnThrGluAspAsnThrAspTyrThrSer

1270 1280 1290 1300 1310 1320

GCTGCTTACAAGCTGTATCGCAACTACGACGGCAATAATCGGGTTCGGCTCGATCAA
AlaAlaTyrLysLeuTyrArgAsnTyrAspGlyAsnLysSerGlyPheGlySerIleLys

1330 1340 1350 1360 1370 1380

GTGGACGCCGCTACGTCCGATACGGAGAACAGCTCGGTATACGCTTCGGTAACTGACGAG
ValAspAlaAlaThrSerAspThrGluAsnSerSerValTyrAlaSerValThrAspGlu

1390 1400 1410 1420 1430 1440

GAGAATTCCGAACCTCCACCTGATCGTGCTGAATAAAATTTCGACGATCCGATCAACGCT
GluAsnSerGluLeuHisLeuIleValLeuAsnLysAsnPheAspAspProIleAsnAla

Fig. 15C

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1450 1460 1470 1480 1490 1500

ACTTTCCAGCTGTCTGGTGATAAAACCTACACATCCGGGAGAGTATGGGCTTCGACCAA
 ThrPheGlnLeuSerGlyAspLysThrTyrThrSerGlyArgValTrpGlyPheAspGln

1510 1520 1530 1540 1550 1560

ACCGGATCCGACATTACGGAACAAGCAGCTATAACGAATATTAACAACAATCAATTCAACG
 ThrGlySerAspIleThrGluGlnAlaAlaIleThrAsnIleAsnAsnAsnGlnPheThr

1570 1580 1590 1600 1610 1620

TATACGCTTCCTCCATTGTCGGCTTACCACATTGTTCTGAAAGCGGATAGCACCGAACCG
 TyrThrLeuProProLeuSerAlaTyrHisIleValLeuLysAlaAspSerThrGluPro

1630 1640 1650 1660 1670 1680

GTCATCTCCGAGATCCCCTCCAGCAGCACAGCTCTCCGGTCAACCAGCCTACCGACCC
 ValIleSerGluIleProSerSerSerThrSerSerProValAsnGlnProThrSerThr
 Linker 43 kdal B region

1690 1700 1710 1720 1730 1740

AGCACCAACGTCCACCTCCACCACACCTCGAGCCCCGCCAGTCCAGCCTACGACTCCCAGCGGC
 SerThrThrSerThrSerThrSerProProValGlnProThrThrProSerGly

1750 1760 1770 1780 1790 1800

TGCAC TGCTGAGAGGTGGGCTCAGTGC GGCGGCAATGGCTGGAGCGGCTGCACCA CCTGC
 CysThrAlaGluArgTrpAlaGlnCysGlyGlyAsnGlyTrpSerGlyCysThrThrCys
 43 kdal A region

1810 1820 1830 1840 1850

GTCGCTGGCAGCACTTGCACGAAGATTAATGACTGGTACCATCAGTGC CCTGTAG
 ValAlaGlySerThrCysThrLysIleAsnAspTrpTyrHisGlnCysLeu---

Fig. 15D

INTERNATIONAL SEARCH REPORT

International Application No PCT/DK 91/00124

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC IPC5: C 12 N 9/42, C 12 N 1/14								
II. FIELDS SEARCHED <table style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th colspan="2" style="text-align: center; padding-bottom: 2px;">Minimum Documentation Searched⁷</th> </tr> <tr> <th style="width: 15%;">Classification System</th> <th style="width: 85%;">Classification Symbols</th> </tr> </thead> <tbody> <tr> <td style="padding-top: 2px;">IPC5</td> <td style="padding-top: 2px; text-align: center;">C 12 N</td> </tr> </tbody> </table>			Minimum Documentation Searched ⁷		Classification System	Classification Symbols	IPC5	C 12 N
Minimum Documentation Searched ⁷								
Classification System	Classification Symbols							
IPC5	C 12 N							
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched ⁸								
SE,DK,FI,NO classes as above								
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹								
Category	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³						
X	GENE, Vol. 51, 1987 Tuula T. Teeri: "Homologous domains in Trichoderma reesei cellulolytic enzymes: gene sequence and expression of cellobiohydrolase II", see page 43 - page 52 especially page 46 --	1-23						
A	US, A, 4435307 (BARBESGAARD ET AL) 6 March 1984, see the whole document --	1-23						
A	Chemical Abstracts, volume 105, no. 13, 29 September 1986, (Columbus, Ohio, US), Rao, Mala et al: "Purification characterization, and synergistic action of endoglucanases from Fusarium lini ", see page 307, abstract 110894p, & Biotechnol.Bioeng. 1986, 28(7), 1100-1105 --	1-23						
* Special categories of cited documents: ¹⁰ "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed								
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IV. CERTIFICATION								
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report							
20th August 1991	1991 -08- 29							
International Searching Authority	Signature of Authorized Officer							
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Yvonne Siösteen								

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	Chemical Abstracts, volume 109, no. 23, 5 December 1988, (Columbus, Ohio, US), Hayashida, Shinsaku et al: "Cellulases of <i>Humicola insolens</i> and <i>Humicola grisea</i> ", see page 295, abstract 207112c, & <i>Methods Enzymol.</i> 1988, 160(), 323-332 --	1-23
A	Chemical Abstracts, volume 105, no. 3, 21 July 1986, (Columbus, Ohio, US), Hayashida, Shinsaku et al: "Production and characteristics of Avicel-disintegrating endoglucanase from a protease-negative <i>Humicola grisea</i> var, <i>thermoidea</i> mutant", see page 316, abstract 20820g, & <i>Appl. Environ. Microbiol.</i> 1986, 51(5), 1041-1046 --	1-23
A	Chemical Abstracts, volume 103, no. 7, 19 August 1985, (Columbus, Ohio, US), Rodionova, N.A. et al: "Characterization of endo-1,4-beta-glucanases of <i>Geotrichum candidum</i> ", see page 237, abstract 50208q, & <i>Prikl.Biokhim.Mikrobiol.</i> 1985, 21(3), 309- 317 -----	1-23

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**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.PCT/DK 91/00124**

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the Swedish Patent Office EDP file on **91-06-27**
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A- 4435307	84-03-06	AT-B- 384442 BE-A- 888632 CH-A- 663511 DE-A- 3117250 FR-A-B- 2481712 GB-A-B- 2075028 JP-C- 1443072 JP-A- 57023699 JP-B- 61016316 NL-A- 8102123	87-11-10 81-10-29 87-12-31 82-04-01 81-11-06 81-11-11 88-06-08 82-02-06 86-04-30 81-11-16

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